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Brk tyrosine kinase signalling in the gastrointestinal tract

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Zusammenfassung

Für viele entwicklungsbiologische Prozesse ist die Tyrosin Kinase vermittelte Signaltransduktion von entscheidender Bedeutung. Die Tyrosin Kinase Brk (breast tumor kinase) stellt den Prototypen nicht N-terminal myristoylierter, Nicht-Rezeptor Tyrosin Kinasen dar. Die Expression dieser intrazellulären Kinase ist auf epitheliale Gewebe beschränkt und wird während der Entwicklung differentiell reguliert, wobei höchste RNA- und Proteinniveaus im Darmepithel detektiert wurden. In normalen Geweben ist die Brk Expression auf nichtproliferierende, terminal differenzierte Zellen beschränkt. Zusätzlich zu der entwicklungsregulierten und zelltypspezifische Expression in normalen Geweben, wurde eine Überexpression dieser Kinase in einigen epithelialen Tumoren festgestellt. Die genaue biologische Funktion dieser Tyrosin Kinase ist bis heute unbekannt. In der vorliegenden Arbeit wurde der Einfluss von Brk auf die Eigenschaften des murinen Darmepithels untersucht.

Um die regulatorische Funktion von Brk im murinen Darmepithel zu untersuchen, wurde das *brk* Gen in der Maus mittels homologer Rekombination unterbrochen. Brk knockout Mäuse zeigten keine offensichtlichen Defekte in ihrer Entwicklung jedoch eine erweiterte Proliferationszone in den Krypten des Darmepithels und verlängerte Villi im Bereich des Dünndarms. Eine genauere Untersuchung zeigte, dass die Inaktivierung von Brk zu einer erhöhten Akkummulation von nukleärem β-catenin in den Krypten der knockout Mäuse führte. Ausserdem konnte eine Hochregulierung des β-catenin Zielgens *c-myc* in den Krypten des Dünn- und Dickdarms festgestellt werden. Zusätzlich zeigten Brk knockout Mäuse eine Aktivierung des Akt-Signaltransduktionswegs und damit eine vermutliche Induktion von Überlebenssignalen in ihrem Darmepithel. Diese Ergebnisse zeigen, dass die Inaktivierung der Brk Tyrosine Kinase zu einer Störung des Fliessgleichgewichts zwischen Zellproliferation und –differenzierung mit aberranter Signaltransduktion im Darmepithel führt.

Weiterhin traten bei Brk knockout Mäuse chronische Entzündungen des Darmepithels sowie eine erhöhte Sensibilität gegenüber dem Reizmittel DSS auf. Im Gegensatz dazu, zeigten Wildtyp Mäuse eine mit der Literatur übereinstimmende Reaktion zu DSS. Interessanterweise wurde zusätzlich eine Induktion der Brk Expression im Darmepithel behandelter Wildtyp Mäuse vorgefunden. Diese

Ergebnisse verdeutlichen das durch die Abwesenheit der Brk Tyrosin Kinase verursachte Ungleichgewicht in der Homöostase des Darmepithels. Ausserdem lassen sie eine schützende Funktion dieser Kinase in Entzündungsprozessen vermuten.

Apoptose - programmierter Zelltod - spielt eine grosse Rolle in der Entwicklung und Aufrechterhaltung der Integrität des Darmepithels. Die Deregulation dieses Prozesses hat häufig krankhafte Erscheinungen wie Krebs zur Folge. Trotz einer Aktivierung des Akt-Signaltransduktionswegs in Brk knockout Mäusen wurden keine Unterschiede im Apoptoseniveau unbehandelter Brk knockout und Wildtyp Mäuse gefunden. Die Behandlung dieser Mäuse mit γ-Strahlung resultierte jedoch in einer signifikanten Beeinträchtigung der Apoptosereaktion in knockout Mäusen. Im Gegensatz zu Wildtyp Mäusen waren Brk knockout Mäuse resistent gegenüber Bestrahlung, was die Anhäufung onkogener Mutationen und damit die Entwicklung von Krebs fördert. Ausserdem wurde erneut eine Induktion der Expression des Brk-Proteins im Darmepithel behandelter Wildtyp Mäuse festgestellt, was eine schützende Funtion dieser Kinase im Darmepithel weiter unterstreicht.

Zusammenfassend kann gesagt werden, dass die Brk Tyrosin Kinase eine entscheidende Rolle in der Aufrechterhaltung der Homöstase und Integrität des Darmepithels spielt. Insbesondere scheint Brk als entscheidender Faktor zur Bestimmung der Sensitivität epithelialer Zellen zu genotoxischem Stress zu fungieren. Entgegen der bisher vermuteten onkogenen Funktion in epithelialen Tumoren scheint Brk im normalen murinen Darmepithel “Tumor Suppressor” ähnliche Funtionen innezuhaben.

Schlagwörter: Brk, Sik, PTK6, Tyrosine Kinase, Darmepithel

Abstract

The **Br**east tumor **k**inase Brk is a prototypical non-myristoylated, non-receptor tyrosine kinase. Brk expression is epithelial-specific with the highest level of expression in the gastrointestinal tract. In normal tissues, Brk expression is developmentally regulated, and is restricted to cells exiting the cell cycle and undergoing terminal differentiation. Interestingly, Brk expression is upregulated in some epithelial tumors. To date, the biological role of this intracellular tyrosine kinase in differentiation and oncogenesis remains poorly understood. The present study is focused on gaining a better understanding of the physiological role of Brk in the gastrointestinal tract.

To determine the role of Brk in the gastrointestinal tract, we disrupted mouse *brk* by homologous recombination. Loss of Brk in the mouse resulted in increased intestinal epithelial cell turnover and the appearance of longer small intestinal villi suggesting a role for Brk in the maintenance of intestinal tissue homeostasis. Brk deficient mice displayed enhanced accumulation of nuclear β-catenin and upregulation of the β-catenin target gene *c-myc* in the crypt compartment of small and large intestine. In addition, Brk deficient mice exhibited increased Akt kinase activity, suggesting an increase in pro-survival signaling. Furthermore, chronic inflammation was observed in Brk deficient mice, and they showed increased susceptibility to a colon injury model utilizing DSS. Interestingly, wild-type mice exhibited a significant upregulation of nuclear Brk protein throughout the intestinal epithelium in response to DSS.

Even though Brk deficient mice showed increased Akt pro-survival signaling, there was no corresponding difference in base-line apoptosis in untreated wild-type and knockout animals. However, subjected to γ-irradiation, Brk deficient animals were significantly impaired in the apoptotic response, a crucial mechanism protecting the stem cells in the small intestinal crypt against the accumulation of oncogenic mutations and the development of cancer. Wild-type mice, however, exhibited normal levels of apoptosis following γ-irradiation accompanied by a rapid induction of Brk expression in crypt cells. The ability of Brk to sensitize cells to apoptotic stimuli was also observed *in vitro* in Rat1A fibroblasts overexpressing the kinase.

These recent findings suggest that Brk plays a crucial role in the maintenance of intestinal tissue homeostasis and integrity. In addition, Brk may function to protect the intestinal epithelium against DNA-replication-induced errors and hence the development of cancer. Contrary to reported oncogenic properties of Brk in other epithelial tissues, Brk appears to have tumor suppressor-like functions in the mouse gastrointestinal epithelium.

Keywords: Brk, Sik, PTK6, Protein tyrosine kinase, intestine

Abbreviations

ACF	Aberrant Crypt Focus
AOM	Azoxymethane
ATP	Adenosine Triphosphate
BrdU	5-Bromo-2-deoxyuridine
Brk	Breast Tumor Kinase
BSA	Bovine Serum Albumin
CD	Crohn's Disease
CIN	Chromosomal Instability
CRC	Colorectal Cancer
DAB	3,3'-Diaminobenzidine Tetrahydrochloride
DAPI	4',6-Diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DSS	Dextran Sodium Sulfate
DTT	Dithiothreitol
EDTA	Ethyleneediamine tetraacetate
ES cells	Embryonic Stem Cells
FAP	Familial Adenomatous Polyposis
FBS	Fetal Bovine Serum
HNPCC	Hereditary Nonpolyposis Colorectal Cancer
IBD	Inflammatory Bowel disease
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
KH	Heteronuclear Ribonucleo-protein K Homology
M	Molar
MIF	Migration Inhibition Factor
MIN	Multiple Intestinal Neoplasia
MMR	Mismatch Repair
neo	Neomycin Phosphotransferase
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen

PCR	Polymerase Chain Reaction
PTK	Protein Tyrosine Kinase
PVDF	Polyvinylidene Difluoride
RBM	RNA-Binding Motif
RNA	Ribonucleic Acid
RTK	Receptor Tyrosine Kinase
SDS	Sodium Dodecyl Sulfate
SH	Src Homology Domain
Sik	Src-related Intestinal Kinase
STAR	Signal Transducers and Activators of RNA
tRNA	Transfer Ribonucleic Acid
UC	Ulcerative Colitis

1 Introduction

1.1. Protein tyrosine kinases in signal transduction

Protein tyrosine kinases (PTKs) are a large and diverse multigene family evolved to perform functions that regulate a range of cellular processes, including cell growth, differentiation, death, motility, adhesion, and cell-to-cell communication (Pawson, 1994). They consist of a group of very closely related protein kinases, which are distinguished by common sequence motifs and tyrosine-specific catalytic activity that differs from the broader group of serine/threonine kinases (Robinson, et al., 2000, Manning, 2002 #3600). While members of the protein kinase superfamily are found throughout all kingdoms of life, tyrosine kinases are restricted to metazoan cells. Tyrosine phosphorylation is a hallmark of metazoans and is associated with a variety of cellular processes directly or indirectly linked to environmental clues and related to the multicellular status of the organism. Dysfunction of cellular phosphorylation is associated with a variety of human diseases, from cancer to diabetes (Hunter, 2002). Each PTK possesses a functional kinase domain capable of catalyzing the transfer of the gamma-phosphate group of ATP to the hydroxyl groups of specific tyrosine residues in peptides. Although phosphotransfer reactions catalyzed by various PTKs are similar with regard to their basic mechanisms, the recognition of substrates by PTKs and, therefore, subsets of proteins phosphorylated by them show a considerable degree of specificity.

Aberrant kinase activity is implicated in a variety of human diseases, in particular those involving inflammatory or proliferative responses, such as cancer. Directly or indirectly, more than 400 human diseases have been connected to protein kinases. The ability to modulate kinase activity therefore represents an attractive therapeutic strategy for the treatment of human illnesses. However, despite a wealth of potential targets, only a handful of kinases are targeted by drug compounds currently on the market.

The PTK superfamily can be divided into two groups according to the presence of transmembrane and extracellular domains, which enable PTKs possessing them to recognize extracellular ligands, in particular, various peptide growth factors. Specific ligands and intracellular signaling pathways induced by them have been identified for many, albeit not for all, membrane-spanning PTKs (Schlessinger, 2000).

PTKs lacking the transmembrane and extracellular sequences are referred to as non-receptor or non-transmembrane PTKs. Thirty-two genes encoding for non-receptor PTKs clustered into 10 families are present in the human genome (Robinson, et al., 2000, Manning, 2002 #3600).

1.2. Brk family tyrosine kinases

Brk protein tyrosine kinases belong to a novel family of intracellular soluble tyrosine kinases distinct from Src family kinases. This family branched off the Src tyrosine kinase family tree early in evolution. Although developmental expression patterns and functional overexpression in vitro have associated these kinases with growth suppression and differentiation, their physiological functions remain largely unknown.

The Brk family of non-receptor PTKs has four members: Brk, Frk, Srms, and Src42A (Serfas and Tyner, 2003). They are defined by a highly conserved exon structure that is distinct from other major intracellular tyrosine kinase families including c-Src (Lee, et al., 1998). Brk and Frk have been cloned independently from human, mouse, and rat cells by several laboratories, and there are multiple names for these PTKs; Brk is also known as PTK6 and Sik (Lee, et al., 1993; Mitchell, et al., 1994; Vasioukhin, et al., 1995), whereas Frk is also known as Rak, Bsk, Iyk, and Gtk (Cance, et al., 1994,; Lee, et al., 1994; Oberg-Welsh and Welsh, 1995; Sunitha and Avigan, 1996; Thuveson, et al., 1995). Srms was cloned and studied only in mice, but its ortholog is present in the human genome, as well (Kohmura, et al., 1994). Src42A, also known as Dsrc41, was cloned and studied only in *Drosophila*, and it shares 61% amino acid identity with its putative mammalian ortholog Frk (Serfas and Tyner, 2003; Shishido, et al., 1991). Brk and Frk are expressed specifically in epithelial cells, primarily those of the intestinal tract, and their expression is upregulated in some epithelial tumors. In contrast, Srms expression is ubiquitous, although found most abundantly in lung, liver, spleen, kidney and testis (Kohmura, et al., 1994). Src42A is expressed in a wide range of tissues during embryonic development.

Brk-family PTKs are highly homologous to Src-family PTKs, even more so than are Csk-family PTKs (Robinson, et al., 2000). Their domain structure is very similar to that of Src-family PTKs, consisting of a highly divergent N-terminal

sequence followed by an SH3 domain, an SH2 domain, and a tyrosine kinase domain. SH2 and SH3 domains bind to phosphorylated tyrosine residues and proline rich sequences of target proteins respectively (Songyang, et al., 1993, Cohen, 1995 #3503). As found with Src family PTKs, these domains are involved in both intermolecular associations that regulate signaling cascades, and intramolecular associations that autoregulate protein kinase activity (Sicheri and Kuriyan, 1997; Thomas and Brugge, 1997; Xu, et al., 1997). However, unlike Src-family PTKs, most Brk-family PTKs lack the N-myristoylation site and are thus not specifically targeted to the membrane. The only exception from this rule is rodent Frk, which retains the glycine residue in position 2 and is consequently myristoylated and localized to the membrane (Sunitha and Avigan, 1996). Nuclear localization of Brk and Frk has been reported (Cance, et al., 1994, Derry, 2000 #2416; Haegebarth, et al., 2004). Frk, Brk and Src42A, although not Srms, possess tyrosine residues near their C-termini, which might mediate negative regulation of these PTKs in an Src-like fashion. Frk and Src42A have been shown to be phosphorylated by Csk and dCsk respectively (Cance, et al., 1994; Read, et al., 2004).

Overexpression of the epithelial specific tyrosine kinase Frk in a number of cell lines of epithelial and mesenchymal origin resulted in potent growth arrest that may function, in part, through its interaction with pRb (Craven, 1995 #3330; Oberg-Welsh, 1998 #3506; Anneren, 2000 #3537}. Frk has been shown to promote neurite outgrowth in PC12 cells (Anneren, et al., 2000), and is also able to associate with and phosphorylate SHB, the Src homology 2 (SH2) domain adaptor protein, thus regulating versatile signal transduction pathways involved in cell survival, differentiation, and proliferation (Anneren, 2002; Anneren, et al., 2003). Recent studies showed that expression of Frk is able to block breast ductal carcinoma cell proliferation at the G1 phase of the cell cycle (Meyer, et al., 2003), consistent with findings that its expression is progressively lost from human breast tumors (Berclaz, et al., 2000). Furthermore, Frk is involved in the apoptotic response of B-cells to inflammatory cytokines (Anneren and Welsh, 2001) (Welsh, et al., 1999). These findings hinted that Frk might be involved in the regulation of cell differentiation. However, Frk-deficient mice demonstrated no morphological abnormalities in epithelial tissues, no related metabolic or developmental changes, and no increase in the incidence of spontaneous tumors (Chandrasekharan, et al., 2002). The only

phenotypic change observed in these mice was a slight decrease in the level of circulating thyroid T3 hormone.

Similarly to Frk, homozygous deletions of Srms in mice have been produced, with no detectable phenotypic effect (Kohmura, et al., 1994). Furthermore, mice deficient for Brk are viable and fertile, suggesting redundant functions for the mammalian Brk family members (Wenjun Bie, 2005). In contrast, loss of function alleles of Src42A cause homozygous lethality in *Drosophila* (Lu and Li, 1999). Src42A has been identified as a negative regulator of Ras pathway receptor tyrosine kinase signaling (Lu and Li, 1999). Furthermore, Src42A has been show to activate the Bsk signaling pathway in epidermal closure, the *Drosophila* homolog to Jnk pathway signaling (Tateno, et al., 2000).

1.3. The non-receptor tyrosine kinase Brk

The intracellular tyrosine kinase Brk was identified in a screen for protein tyrosine kinases involved in breast cancer (Mitchell, et al., 1994), from the mouse small intestine in a screen for factors that regulate epithelial cell differentiation (Siyanova, et al., 1994), and from cultured human melanocytes (Lee, et al., 1993). Brk expression is restricted to epithelial cells of the skin, gastrointestinal tract, and prostate, with highest levels being expressed in the gastrointestinal tract (Derry, et al., 2003; Llor, et al., 1999; Vasioukhin, et al., 1995). Furthermore, Brk expression is developmentally regulated. It is detected late in gestation in the mouse, at mouse embryonic day 15.5 in the differentiating granular layer of the skin and at embryonic day 18.5 in the differentiating intestine (Vasioukhin, et al., 1995). Brk expression is initiated as cells migrate away from the proliferative zone and begin the process of terminal differentiation. Overexpression of Brk in mouse keratinocytes resulted in increased expression of the differentiation marker filaggrin during calcium-induced differentiation (Vasioukhin and Tyner, 1997). Brk is expressed in many breast carcinoma cell lines and primary breast tumors, but has not been detected in normal human breast tissue (Barker, et al., 1997; Mitchell, et al., 1994), or at any stage of mammary gland differentiation in the mouse (Llor, et al., 1999). Modest increases in Brk levels have been detected in colon tumors and Brk expression increases during differentiation of Caco-2 colon adenocarcinoma cells (Llor, et al., 1999).

While Brk resembles Src structurally with SH3 and SH2 protein-protein binding domains, the tyrosine kinase domain, and the regulatory C-terminus, it lacks the amino-terminal myristoylation signal that localizes Src to the cell membrane, and therefore is not specifically targeted to the membrane (Fig. 1) (Vasioukhin, et al., 1995). Its intracellular localization is flexible and it can be found to be present in the nucleus as well as the cytoplasm or at the membrane (Haegebarth, et al., 2004). The Src homology 3 (SH3) domain is involved in intramolecular interactions that regulate kinase activity, interactions with substrates, cellular localization, and association with other protein targets (Pawson, 1995). SH3 domains bind proline-rich sequences of the consensus PXXP in substrate proteins. The SH2 domain on the other hand is essential in controlling interactions. It recognizes and binds to phosphorylated tyrosine residues, with the specificity being determined by the 3-5 amino acids following the tyrosine residue (Songyang, et al., 1993).

Like Src family members, the SH3 and SH2 domains of Brk engage in intramolecular interactions with the kinase domain to form an autoinhibited conformation (Qiu and Miller, 2002). Brk activity is negatively regulated by tyrosine phosphorylation of its C-terminal tyrosine residue, Tyr-447 in mouse, similar to that of Src-family PTKs (Fig. 1). However, it remains to be determined how this tyrosine becomes phosphorylated in Brk, since it is phosphorylated neither by Brk itself nor by Csk (Qiu and Miller, 2002). Csk is playing this role for Src-family PTKs (Liu, et al., 1993). Phosphorylation on Tyr-447 in Brk causes the intramolecular interaction of this tyrosine with the SH2 domain, which induces the binding of the SH3 domain to the linker region connecting the SH2 domain and the kinase domain, accompanied by the binding of the linker region to the kinase domain. These intramolecular interactions prevent the binding of ATP to the critical catalytic residues rendering Brk inactive. Mutation of the carboxy-terminal tyrosine of Brk to phenylalanine (Y447F) results in increased enzyme activity when overexpressed in epithelial cells, supporting a role for this residue in autoinhibition (Derry, et al., 2000; Qiu and Miller, 2002). However in contrast to increasing its transforming potential, mutation of the Brk regulatory tyrosine resulted in a decrease in the ability of Brk to induce anchorage–independent growth of fibroblasts (Kamalati, et al., 1996).

Analysis of Brk using mutagenesis, mass-spectrometry and enzyme kinetics indicated that Brk is capable of autophosphorylation, which significantly upregulates

its kinase activity (Qiu and Miller, 2002). This study mapped the autophosphorylation site of Brk to Tyr-341, a conserved tyrosine residue inside the activation loop (Fig. 1). Autophosphorylation of Tyr-341 in an intermolecular process causes displacement of this tyrosine from a hydrophobic pocket of the PTK catalytic domain, resulting in the correct positioning of all key catalytic residues and the formation of the substrate binding surfaces, thus leading to the full activation of the enzyme.

Thus, physiological regulation of Brk is mediated by modulation of the interactions described above, achieved by (a) phosphorylation/dephosphorylation of the C-terminal regulatory site, and (b) binding of the SH2 and SH3 domains of Brk to various phosphotyrosine- or polyproline-containing proteins.

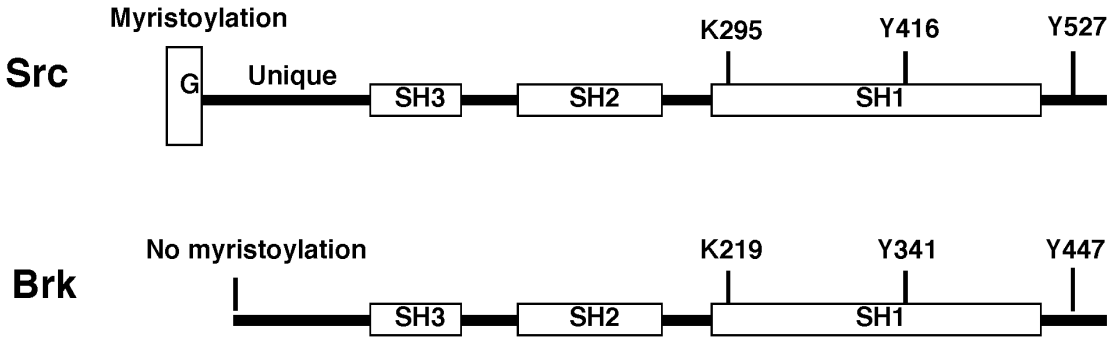

Fig. 1: Structure of Src and Brk tyrosine kinases. Src and Brk tyrosine kinases share 44% amino acid identity. Both Src and Brk proteins contain SH3 and SH2 domains that regulate protein-protein interactions as well as a conserved catalytic SH1 domain. The tyrosine at 527 in Src and at 447 in Brk regulates kinase activity. Phosphorylation on these tyrosine residues results in the intramolecular formation of an inactive conformation involving both SH2 and SH3 domains. The lysine at 295 in Src and at 219 in Brk correlates with the ATP binding site and its mutation results in a dominant-negative protein. Tyrosines 416 in Src and 341 in Brk reside in the activation loop and are autophosphorylated resulting in increased kinase activity. In contrast to Src, Brk lacks an aminoterminal consensus myristoylation sequence.

Like Src, localization of the Brk tyrosine kinase has been correlated with its activities. Brk was localized in the nucleus of normal luminal prostate epithelial cells as well as of well-differentiated but less tumorigenic prostate cancer cells, but it was a predominantly cytoplasmic protein in poorly differentiated tumors and more aggressive tumor cell lines (Derry, et al., 2003). Thus, relocalization of the Brk kinase during development of prostate cancer may indicate the disruption of a signaling pathway important for maintaining the normal phenotype of prostate epithelial cells.

In addition, a correlation between tumorigenicity and the subcellular localization of Brk has also been found in oral squamous cell carcinomas (OSCC) (Petro, et al., 2004). Brk was present in the nucleus and cytoplasm of normal oral epithelium (NOE) and moderately differentiated OSCC cells. However, in poorly differentiated OSCC cells, Brk was localized in perinuclear regions, supporting the notion that subcellular localization of this tyrosine kinase plays a role in determining its growth regulating functions.

1.4. Brk signaling substrates

Although the exact biological function of the Brk tyrosine kinase is still largely unknown, progress has been made in identifying substrates of this PTK. Brk has been found in complexes with EGF-R (Kamalati, et al., 1996), GTPase activating protein-associated p65 (Vasioukhin and Tyner, 1997), the putative adaptor protein Bks (Mitchell, et al., 2000), the EGF-R family member erbB3/HER3 (Kamalati, et al., 2000), the RNA-binding proteins Sam68, SLM-1 and SLM-2 (Derry, et al., 2000; Haegebarth, et al., 2004), the serine-threonine kinase PKB/Akt (Zhang, et al., 2004), and the focal adhesion protein paxillin (Chen, et al., 2004).

Like c-Src, overexpression of Brk has been shown to sensitize mammary epithelial cells to EGF and to induce transformation of fibroblasts (Kamalati, et al., 1996). The first is likely mediated by the functional interactions of Brk with the epidermal growth factor receptor-related receptor ErbB3, which enhance EGF signaling via the phosphoinositide 3-kinase/Akt pathway (Kamalati, et al., 2000). The use of RNA interference to efficiently and specifically downregulate Brk protein levels in breast carcinoma cells resulted in a significant suppression of their proliferation. Additionally, through the expression of a kinase-inactive mutant, it was shown that Brk can mediate promotion of proliferation via a kinase-independent mechanism, potentially functioning as an adaptor (Harvey and Crompton, 2003). Evidence for a direct interaction between Brk and Akt/Protein kinase B (PKB) has recently been reported (Zhang, et al., 2004). In unstimulated cells, Brk has been shown to associate with and tyrosine-phosphorylate Akt, and herein to inhibit Akt kinase activity and downstream signaling. It has been proposed that Brk kinase activity normally limits basal or “steady state” activity of Akt in resting cells (Zhang,

et al., 2004). However, upon stimulation with EGF and subsequent PI3-K activation, the Brk-Akt complex dissociates resulting in activation of Akt signaling. Taken together with the inhibition of receptor tyrosine kinase signaling by Brk family members (Serfas and Tyner, 2003; Zhang, et al., 1999), the biological function of this PTK in normal epithelial cells may be to limit the magnitude of growth factor receptor inputs to multiple intracellular signaling pathways.

Brk has been shown to phosphorylate the RNA-binding proteins Sam68 and the two Sam68-like mammalian proteins SLM-1 and SLM-2, and negatively regulate their RNA-binding activities (Derry, et al., 2000; Haegebarth, et al., 2004). Sam68, SLM-1 and SLM-2 are members of the STAR family of KH (heteronuclear ribonucleoprotein K homology) domain containing RNA binding proteins that regulate different aspects of RNA metabolism, including transport, stability, translation and processing (Lasko, 2003; Lukong and Richard, 2003; Vernet and Artzt, 1997). Although Sam68 can be phosphorylated by other intracellular tyrosine kinases, only Brk has been shown to colocalize with Sam68 in the nucleus where it phosphorylates Sam68, resulting in inhibition of its ability to bind RNA and to function as a cellular Rev homologue (Derry, et al., 2000). Brk further regulates the ability of Sam68 to regulate utilization of specific RNAs in the cytoplasm (Coyle, et al., 2003). Sam68 has also been implicated in a number of cellular processes including transcription, RNA splicing and export, translation, signal transduction, cell cycle progression, and replication of the human immunodeficiency virus and poliovirus (McLaren, et al., 2004; Reddy, et al., 1999; Soros, et al., 2001).

SLM-1 shares many similarities with Sam68; it interacts with many of the same proteins and is also tyrosine phosphorylated by Src during mitosis (Di Fruscio, et al., 1999). SLM-2 was also identified by its ability to interact with RNA-binding motif (RBM) in spermatogenesis and named T-STAR or ETOILE (Venables, et al., 1999). It can regulate the selection of alternative splice sites (Stoss, et al., 2001). Both proteins are tyrosine phosphorylated by Brk, which in turn inhibits their RNA binding abilities (Haegebarth, et al., 2004). The RNA-binding functions of these STAR proteins have been implicated in the posttranscriptional regulation of gene expression. Tyrosine phosphorylation and subsequent inhibition by Brk may regulate specific STAR family signaling pathways.

Inhibition of Sam68's RNA binding ability has been shown to result in reduced levels of cyclin D1 and inhibition of cell proliferation (Barlat, et al., 1997). It was proposed that in normal epithelial tissues, Brk is positioned to inhibit RNA-binding activities of its nuclear STAR protein family substrates during differentiation (Haegebarth, et al., 2004). Signal transduction pathways have recently been shown to regulate gene expression not only by modifying the activity of transcriptional regulators, but also at the RNA level by posttranscriptional mechanisms (Lasko, 2003; Matter, et al., 2002). Thus, Brk appears to integrate external signals and gene expression regulation by its specific regulation of STAR family signaling pathways.

Recently, paxillin has been identified as a binding partner and substrate of Brk (Chen, et al., 2004). Upon EGF stimulation Brk is catalytically activated resulting in tyrosine phosphorylation of paxillin. This in turn promotes the activation of small GTPase Rac1 via the function of CrkII. Through this pathway, Brk is capable of promoting cell motility and invasion, and functions as a mediator of EGF-induced migration and invasion. This provides the first potential link between Brk and metastatic malignancy (Chen, et al., 2004).

1.5. Morphology and homeostasis of the intestinal epithelium

The non-receptor Brk tyrosine kinase shows a highly specific expression pattern in regenerating epithelial linings with highest levels being expressed in the gastrointestinal tract. Its expression is restricted to non-proliferating cells of the villus, suggesting a role for this tyrosine kinase in the regulation of proliferation and differentiation in the intestinal epithelium (Llor, et al., 1999; Vasioukhin, et al., 1995).

The gastrointestinal tract constitutes a tube consisting of three tissue layers – the smooth muscle layer on the outside, the stromal layer in the middle, and the columnar epithelial cell layer on the inside. It can be morphologically and functionally divided into the small and large intestine. The small intestine is further divided into duodenum, jejunum and ileum (Fig. 2A). It is characterized by its complex organization into villi – finger-like projections into the lumen of the intestine, that dramatically increase the absorptive surface area, – and the crypts of Lieberkühn – bottle-shaped invaginations into the submucosa (Fig. 2B). The large intestine on the other hand has only crypts, and instead of villi there is a flat surface

epithelium (Fig. 2C). The epithelium of the intestine is a constantly regenerating tissue with well-defined zones of proliferation and differentiation. Gradients in gene expression are established within the epithelium in two dimensions: the vertical, crypt to villus dimension, and the horizontal, duodenum to colon dimension. Furthermore, changes in gene expression are also observed in a temporal dimension – from the developing stage to adulthood.

A small number of intestinal stem cells reside near the bottom of each crypt. These cells slowly divide giving rise to a transient population of progenitor cells that rapidly divide and migrate towards the lumen of the intestine. These clonal populations of proliferating progenitor cells give rise to four different cell types – absorptive enterocytes, mucus-secreting goblet cells, and hormone secreting enteroendocrine cells, all of which are populating the villi and are found in both the small and large intestine. The fourth cell type are the Paneth cells, which produce antimicrobial defensins and reside at the crypt bottom of the small intestine but are absent in the colon (Hocker and Wiedenmann, 1998; Porter, et al., 2002).

The crypt progenitor cells are dividing every 12-16 hours, generating about 200 cells per crypt every day (Potten and Loeffler, 1990). Epithelial homeostasis is ensured through 3 mechanisms. First, cells are being continuously shed at the tip of the villi (small intestine) or the surface epithelium (colon) to counterbalance the crypt cell production (Potten, 1998). Second, the cells in the intestinal epithelium are continuously moving upwards with a transit time of approximately 5 days, with the only exception being made by Paneth cells and the immortal stem cell. Paneth cells differentiate as they migrate downwards to the base of the crypt, where they reside for about 20 days before being phagocytized by their neighbors. And third, proliferation is restricted to the crypt niche, thus resulting in the maintenance of distinct proliferative and differentiated compartments (Hermiston, et al., 1996). Deregulation of the crypt homeostasis is a feature of neoplastic transformation, and is evident in the earliest stage of colon cancer.

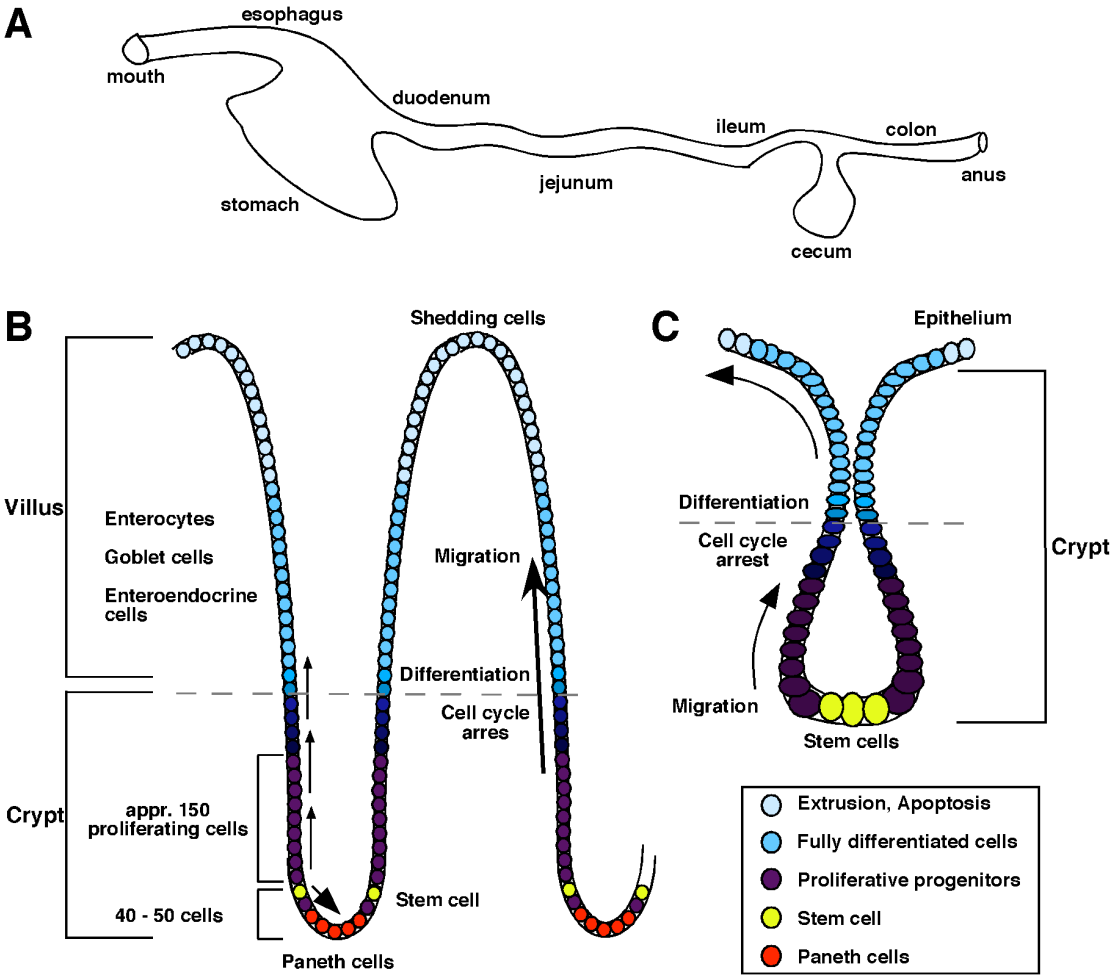

Fig. 2: Schematic presentation of the mammalian digestive tract. (A) Mammalian digestive tract. Digestion starts in the mouth, where food is being macerated and partially digested. The esophagus serves as an alimentary organ, transporting food into the stomach, where it is digested by various enzymes. Most of the nutrient absorption occurs in the small intestine whereas water is absorbed mainly in the large intestine. The cecum serves for the storage of food. (B) Structure of the small intestine. Putative stem cells reside immediately above the Paneth cells. Progenitors stop proliferating at the crypt-villus junction and express differentiation markers. Enteroendocrine, absorptive, and mucus-producing cells migrate upward, whereas Paneth cells migrate downward and localize at the bottom of the crypt. (C) Structure of the large intestine. Stem cells reside at the crypt bottom. Progenitors are amplified by constant division along the bottom two thirds of the crypts, whereas cell cycle arrest and differentiation occur when progenitors reach the top third of the crypts. Paneth cells are absent in the colon.

1.6. Intestinal neoplasia

Colorectal cancer (CRC) is the second most common type of cancer with one million new cases diagnosed per year worldwide. Approximately 5% of the Western population will develop colorectal malignancies during their lifetime (Jemal, et al., 2002). Patients with a familial risk make up approximately 20% of all patients with CRC, whereas approximately 5-10% are inherited in an autosomal-dominant fashion (Lynch and de la Chapelle, 2003). Genomic instability is a characteristic of all intestinal malignancies.

Hereditary cancer can be divided into two categories based on the presence of polyposis, as exemplified by familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). Patients with FAP develop large numbers of benign adenomatous polyps of the colorectal epithelium early in adulthood (Haggitt and Reid, 1986). Almost invariably, some of these will progress into invasiveness and, ultimately, metastasize. These CRC tumors characteristically display chromosomal instability (CIN) and harbor mutations in various tumor suppressor genes and oncogenes such as *APC*, *K-ras*, and *p53*. They are caused by initial mutations in the tumor suppressor gene *adenomatous polyposis coli* (*APC*), whose inactivation also occurs in a large percentage of sporadic CRC (Kinzler, et al., 1991; Nakamura, et al., 1992).

Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal-dominant cancer syndrome that predisposes to multiple primary cancers without intestinal polyposis. HNPCC tumors are predominantly located in the proximal colon (Jass, 1998), and their hallmark is microsatellite instability (MIN) (Thibodeau, et al., 1993). Mutations in mismatch repair (MMR) genes have been identified in HNPCC families. As a consequence of MIN mutations in the *β-catenin* gene, transforming growth factor receptor beta II (*TGFBR2*) gene, the pro-apoptotic gene *Bax*, as well as the *APC* gene arise (Huang, et al., 1996; Rampino, et al., 1997).

The probability that a colorectal cell will acquire the genetic changes leading to a benign tumor is low, but approximately 50% of the Western populations still develop such a tumor by the age of 70. Sporadic CRC is caused by a series of genetic alterations, which begin with benign lesions and eventually lead to fully metastatic tumors (Fig. 3) (Fearon and Vogelstein, 1990). Colorectal tumors occur as a result of

the mutational activation of oncogenes coupled with the inactivation of tumor-suppressor genes, followed by mutations in several other genes that are required to produce malignant tumors. These genetic alterations occur in a preferred sequence even though the total accumulation of changes determines the tumor's biologic properties. In the adenoma-carcinoma sequence, the smallest identifiable lesion is an aberrant crypt focus (ACF). ACFs are very heterogenic which has led to controversial issues regarding their origin and involvement in CRC (Cheng and Lai, 2003). The majority of malignant dysplastic ACFs bears APC mutations, whereas non-malignant hyperplastic ACFs are proposed to arise from activating mutations in K-RAS (Nucci, et al., 1997). Expansion of dysplastic ACFs gives rise to adenomas, which acquire additional mutations and eventually progress to carcinoma *in situ* (Fig. 3). Colorectal tumors evolve through a series of restriction points, with only those cells acquiring the correct mutational event expanding. However, several signaling pathways are involved in the progression to CRC.

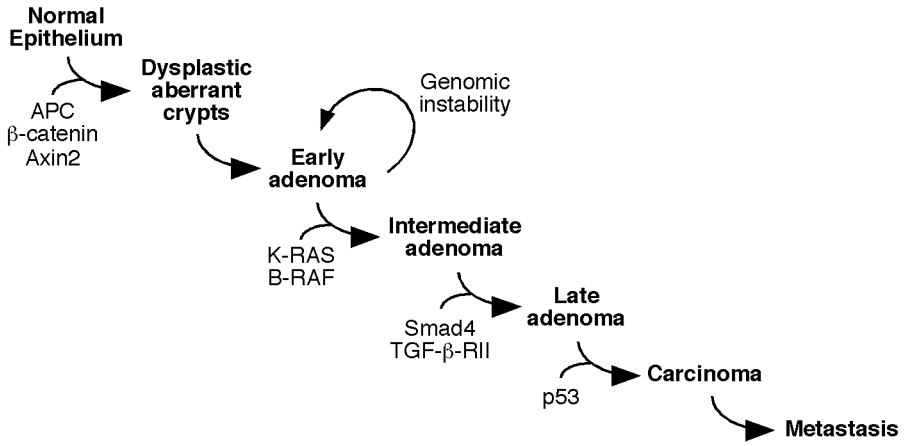

Fig. 3: CRC development. Correlation between CRC progression and the accumulation of genetic alterations according to Fearon & Vogelstein (1990) (Fearon and Vogelstein, 1990).

1.7. Wnt signaling in intestinal development and cancer

The Wnt signaling pathway has been shown to be of major importance in intestinal cell proliferation and stem cell maintenance, and its deregulation leads to malignant development in the mammalian gut epithelium. There are about 20 different secreted Wnt proteins, which bind to about 10 different Frizzled receptors. Downstream targets of Wnt include β-catenin, and the Tcf/Lef transcription factors. Transduction of canonical Wnt signals results in activation of Dishevelled (Dvl) and

inhibition of glycogen synthase kinase 3β (GSK-3β), which in turn causes the stabilization and nuclear translocation of β-catenin. Nuclear β-catenin interacts with members of the Lef/Tcf transcription factor family resulting in activation of target genes (Sancho, et al., 2004). This β-catenin-TCF activity mediates a proliferation/ differentiation switch along the crypt-villus axis (Pinto, et al., 2003; van de Wetering, et al., 2002). Genes whose expression is driven by β-catenin-TCF are present in the proliferating cells of intestinal crypts, while those induced upon cessation of β-catenin-TCF activity are expressed by differentiated cells in the intestinal epithelium. c-Myc has been identified as one of the main mediators of this switch (He, et al., 1998). Furthermore, this gradient of β-catenin-TCF activity imposes restrictions on the migratory behavior of the intestinal epithelial cells by establishing the expression of EphB receptors and their ephrin-B ligands in complementary domains (Batlle, et al., 2002).

In the absence of Wnt signals, β-catenin is found in a multi-protein complex including APC, casein kinase-1 and GSK-3β, which results in phosphorylation and subsequent degradation of β-catenin (Huelsken and Birchmeier, 2001). Consequently, loss of functional APC or stabilizing mutations in β-catenin result in increased levels of nuclear relocalization of β-catenin and constitutive β-catenin signaling (Sansom, et al., 2004). This in turn has been shown to provide the molecular basis for colorectal tumorigenesis (Bienz and Clevers, 2000; Giles, et al., 2003; Polakis, 2000).

1.8. Tyrosine kinase signaling in the gastrointestinal tract

The continuous renewal of the intestinal epithelium is driven by the rapid proliferation and constant differentiation of intestinal crypt cells. Tyrosine kinases have been shown to regulate both proliferation and differentiation, and activity of tyrosine kinases has been reported in the intestinal epithelium (Pawson and Bernstein, 1990; Pawson and Hunter, 1994). During development, the proliferating fetal intestinal epithelium contains high levels of proteins phosphorylated on tyrosine residues, but these levels decrease as maturation proceeds (Maher and Pasquale, 1988). Furthermore, the crypt compartment of the intestinal epithelium has been shown to contain fifteen-fold higher levels of tyrosine phosphorylated proteins than

the villus epithelium, and the majority of the tyrosine kinases appear to be associated with the cytoskeleton (Burgess, et al., 1989).

Higher levels of cytoskeletal associated non-receptor tyrosine kinase Src protein and activity are found in the crypt epithelial cells (Cartwright, et al., 1993), and the specific activity of Src decreases as primary intestinal crypt cells differentiate (Davidson, et al., 1995). Increased levels of Src family kinase activity have been detected in undifferentiated human colon carcinoma cells, and both the activity and abundance of Src decreases as these cells are induced to differentiate *in vitro* (Foss, et al., 1989). Overexpression of Src in immortalized colon cells results in a partially transformed phenotype (Pories, et al., 1998). The specific activity of Src and the related kinase Yes is elevated 5-20 fold in most colon carcinoma cell lines, primary carcinomas, and dysplastic and malignant adenomas (Summy and Gallick, 2003). These results suggest that downregulation of Src activity is important for differentiation and that up-regulation is required for growth and transformation of intestinal epithelial cells.

Activation of Src family kinases in colon cancer may occur through a variety of mechanisms and is frequently a critical event in tumor progression. At least four mechanisms are known to up-regulate Src PTK activity in cells (Brown and Cooper, 1996): (1) mutations within the coding region of the *src* gene, (2) decreased Tyr-527 phosphorylation on Src, (3) subcellular localization of Src and its substrates, and (4) association of Src with other cellular proteins. Mutations of PTKs within their coding regions often result in activated forms of the protein that continually transmit their signal along the pathway in which they participate. The viral form of Src (v-src) has a deletion in the C-terminus that removes the regulatory tyrosine, which leads to a constitutively activated form of the protein. In addition to mutations, overexpression as a result of amplification of PTKs can result in transformation. Compelling evidence indicates that association of Src with other cellular proteins can regulate its activity (Brown and Cooper, 1996). Exactly how Src family kinases contribute to individual tumors remains to be defined, however they appear to be important for multiple aspects of tumor progression, including proliferation, disruption of cell-cell contacts, migration, invasiveness, resistance to apoptosis, and angiogenesis.

1.9. Apoptosis and survival signaling in the intestinal epithelium

The intestinal epithelium is a rapidly renewing tissue in which cell homeostasis is regulated by a balance among proliferation, growth arrest, differentiation and apoptosis. Apoptosis, or programmed cell death, is a cellular response that regulates important processes such as tissue homoeostasis, defense against certain pathogens, and elimination of unwanted cells. In multicellular organisms, mutations in somatic cells affecting critical genes that regulate cell proliferation and survival cause fatal cancers.

The continuous renewal of the intestinal epithelium consists essentially in the production of epithelial cells in the crypts, which differentiate and then migrate toward the apex of the villus or the surface epithelium where they are shed. It was proposed that apoptosis is the main mechanism responsible for the maintenance of appropriate cell numbers by counterbalancing cell division (Potten, 1992). However, it is still unknown if apoptosis or necrosis is responsible for the shedding of cells, and if enhanced rates of apoptosis compensate for high epithelial cell turnover (Merritt, et al., 1995). It has been shown that the anti-apoptotic protein Bcl-2 is expressed in the proliferating crypt cells whereas the pro-apoptotic Bax protein is expressed near the lumen of the large intestine, supporting the idea that apoptosis plays a role in intestinal tissue homeostasis (Vachon, et al., 2001; Vachon, et al., 2000).

Differential involvement of the MEK/Erk and PI3-K/Akt signaling pathways in the regulation of epithelial cell survival has been reported (Gauthier, et al., 2001; Gauthier, et al., 2001). Apoptosis involves the balanced transcription of anti-apoptotic and pro-apoptotic genes, which is regulated by intracellular signal transduction systems. It has been reported that among members of the mitogen-activated protein kinase (MAPK) family, activation of the extracellular signal-regulated kinases (Erk1/2) promotes cell survival, whereas activation of the stress-activated c-Jun N-terminal kinases ($p46/p54^{JNK}$) and $p38^{MAPK}$ induces apoptosis (Wang, et al., 1998; Wang, et al., 1998; Xia, et al., 1995). However, publications also have provided evidence for a protective role of JNK and p38 against apoptosis (Nishina, et al., 1997; Whitmarsh and Davis, 1996; Zechner, et al., 1998). All MAPKs are regulated as part of core signaling modules downstream from the *ras* proto-oncogene. These modules contain three tiers, wherein MAPKs are activated by tyrosine and threonine phosphorylation by MAPK kinases (MAP2Ks), which in turn are activated by serine

and threonine phosphorylation that is catalyzed by MAPK kinases kinases (MAP3Ks) (Chang and Karin, 2001). Erks respond to mitogenic stimuli, whereas JNKs and p38 kinase respond predominantly to cellular stresses or inflammatory cytokines (Seger and Krebs, 1995).

The serine/threonine kinase Akt/Protein kinase B (PKB) is a major downstream effector of growth factor-mediated cell survival. Growth factor signaling through the phosphoinositide 3-kinase (PI3-K)/Akt pathway has emerged as the major mechanism by which growth factors promote cell survival (Marte and Downward, 1997). Phosphorylation of Akt by PI3-K results in full activation of Akt kinase activity and the subsequent regulation of multiple cellular processes, including the transmission of growth factor-dependent survival signals (Coffer, et al., 1998; Marte and Downward, 1997). Akt inhibits mitochondrial release of cytochrome c (Kennedy, et al., 1997) as well as activation of the death receptor pathway (Gibson, et al., 1999). Signaling through the PI3-K/Akt pathway is ordinarily inhibited by the PTEN phosphatase tumor suppressor (Maehama and Dixon, 1998; Stambolic, et al., 1998; Wu, et al., 1998). Deregulation of this pathway is implicated in a wide range of human cancers. Enhanced signaling from receptor tyrosine kinases, inactivating mutations of the PTEN tumor suppressor (Haas-Kogan, et al., 1998; Li, et al., 1998), and amplification of the *PI3-K* and/or *Akt* genes have been reported in various human cancers (Kaufmann and Gores, 2000). All of these changes result in elevated levels of 3-phosphoinositides and enhanced signaling through Akt.

Interestingly, the small intestinal stem cells with their cell cycle time of 12-16 hours and large division potential of about a thousand times during the life span of a laboratory mouse never decline in their proliferative potential and rarely develop carcinogenic mutations (Potten, et al., 2002). In contrast, a relatively high incidence of tumor development is observed in the colon, suggesting a more effective eradication of malignant precursor cells in the small intestine. This is mainly due to the presence of protective mechanisms that ensure the integrity of the small intestinal stem cell genome. Stem cells in the small intestinal crypt are intolerant to genotoxic damage; they do not undergo cell cycle arrest and repair but commit an altruistic p53-dependent cell suicide – apoptosis (Potten and Grant, 1998). This is supported by expression of the pro-apoptotic Bax protein in the small intestinal crypts (Merritt, et al., 1995). Furthermore, small intestinal stem cells have evolved a selective

segregation process to ensure that they retain only the template strands of DNA at division and hence reduce the risk of replication-induced genetic errors (Potten, 2004; Potten, et al., 2002). These two processes provide an extremely effective mechanism to ensure the integrity of the genome, thus providing an explanation as to why cancer occurs so rarely in the small intestine. In contrast, stem cells in the colon express the anti-apoptotic Bcl2 protein, favoring cell survival despite genetic damage. These observations provide insight into the mechanisms contributing to the high rates of colon cancer.

Radiation has proven to be a valuable tool to induce cell death, reproductive sterilization, and regenerative proliferation in the highly polarized epithelium of the small intestine (Potten, 2004). Whole-body γirradiation of mice with doses of about 8 Gγ causes p53-dependent apoptosis in the small intestinal crypts with peaks at 3-6 hours (early apoptosis) and 24 hours (late apoptosis) post treatment (Potten, 2004). About 6 apoptosis-susceptible cells are located in each crypt, which lack repair capacity and undergo cell suicide in response to radiation, thus providing a protective mechanism that removes cells with DNA damage.

1.10. Inflammation and intestinal cancer

Opposing effects of inflammation on cancer have been described. It is widely accepted that acute inflammation usually counteracts cancer development, while chronic inflammation promotes cancer development. Acute inflammation results in immediate production of pro-inflammatory cytokines and chemokines, but this process is self-limiting in that the immediate response usually gives way to production of anti-inflammatory cytokines as healing progresses.

Tumors of the lower bowel are the fourth leading cause of human cancer mortality, together with stomach and liver cancer accounting for more than two million deaths annually. Although only a small percentage of human colorectal carcinomas (CRC) are attributable to inflammatory bowel disease (IBD), patients with Crohn's disease or ulcerative colitis have significantly increased risk of developing CRC and show higher mortality than patients with sporadic CRC (Baisse, et al., 2004; Itzkowitz and Yio, 2004; Munkholm, 2003). While current concepts implicate chronic inflammation as a key element in promoting cancer risk in IBD, a considerable

number of IBD patients have quiescent inflammation (Itzkowitz and Yio, 2004; Seril, et al., 2003), suggesting that several factors and pathways activated in normal epithelial cells during IBD contribute to the increased cancer risk. Although no specific infectious agent has yet been causally linked to IBD, it is generally acknowledged that intestinal bacteria initiate the cascade of events leading to chronic enterocolitis in susceptible individuals.

It is presumed that chronic inflammation promotes rather than initiates carcinogenesis in a non-cell-autonomous fashion. Disruption of epithelial barrier function would lead to direct contact between the intestinal microflora and the immune system, resulting in the induction of mitogenic and anti-apoptotic signals essential for mucosal repair and regeneration. Hyper-proliferative and anti-apoptotic responses, together with chronic oxidative stress and inflammation, then create a tumor-promoting microenvironment in which transformed epithelial cells thrive more optimally. This indirect mechanism – inflammation of the submucosa, induced by direct contact with the intestinal microflora – promotes tumor outgrowth in the overlying epithelium. Thus, cell-cell and cell-extracellular matrix adhesions are important cellular features involved in intestinal tissue homeostasis, and are essential for the intestinal epithelium to function as a physiological and structural barrier. Disruption of cell adhesion to neighboring cells and extracellular matrix leads to villus atrophy, epithelial hyperplasia, loss of normal absorptive function, and an increased risk of tumorigenesis (Philip, et al., 2004).

Interestingly, recent evidence supported an additional complementary mechanism. Inflammatory signaling in epithelial cells has been shown to directly result in their inappropriate survival and transformation (Greten, et al., 2004; Rakoff-Nahoum, et al., 2004). This process is supported by the intestinal microflora, which affects epithelial cell properties. The existence of a symbiotic relationship between the intestinal flora and the intestinal epithelial cells involves a protective mechanism, in which commensal bacteria constitutively activate the NF-κB survival pathway through Toll-like receptors (TLR) expressed on epithelial cells, supporting cell survival and protecting the epithelium from injury (Greten, et al., 2004; Rakoff-Nahoum, et al., 2004). This cell-autonomous cell survival response is actually necessary for intestinal homeostasis. However, when stimulated in transformed epithelial cells, this pathway accelerates tumor development in a cell-autonomous

fashion and complements the non-cell-autonomous inflammation mechanism (Clevers, 2004).

1.11. Mouse models for colorectal cancer

Cancer is a multi-step process that involves the serial mutation of key genes involved in regulating proliferation, differentiation, survival, and invasive properties of cells (Fig. 3) (Vogelstein and Kinzler, 1993). Mouse models are employed to explore basic mechanisms in the progression from infection to malignancy in the gut.

Azoxymethane (AOM) is a potent carcinogen used to induce colon cancer in rats and mice. It is used to identify other candidate mutant genes in the development of CRC as well as to evaluate the efficacy of preventative treatment for azoxymethane-induced carcinogenesis. AOM is a DNA alkylating agent, which induces tumor formation in the distal colon of susceptible rodents. In this model, mice are injected intraperitonally with 10 mg/kg AOM once a week for the duration of 6 weeks and sacrificed 24 weeks after the last injection. At that time, mice exhibit a high incidence of tumors within the distal colon, with the tumor number greatly dependant on the genetic background. This rodent model aids in the identification of possible preventative approaches to human colon cancer (Corpet and Pierre, 2003).

For IBD and IBD-related CRC, several animal models have been reported. The one most widely used is a mouse model with dextran sodium sulfate (DSS) (Okayasu, et al., 1990) -induced injury of the epithelium accompanied by mild inflammation. To this extend, mice are subjected to 3% DSS in the drinking water for 5 days followed by water only for 3 days (recovery). However, this colitis model needs a long period or repeated administration of DSS to induce colitis and colitis-related CRC, and the incidence and/or multiplicity of induced tumors are relatively low (Okayasu, et al., 2002).

Recently, a novel inflammation-related mouse colon carcinogenesis model has been developed (Tanaka, et al., 2003). Mice were given an initial low dose of AOM (10 mg/kg) followed by 1-week exposure to 2% DSS in drinking water. These mice developed large bowel tumors within 20 weeks, which histologically and biologically strongly resemble the human colitis-related disease. Again, different responses of various strains to IBD-like tumorigenesis have been reported, suggesting the influence

of host genetic determinants on the inflammation severity and tumor risk. In general, genetically engineered mice on a C57BL background are more resistant compared with equivalent mutant mice on other genetic backgrounds such as 129/Sv.

1.12. Aim of the study

A large amount of data showing diverse functions for the Brk tyrosine kinase in the regulation of cell proliferation and differentiation has been accumulated. Despite that, the biological role of this PTK in regulating signaling pathways involved in cell proliferation and differentiation crucial in maintaining a healthy organism is still poorly understood. Brk expression has been detected in breast, colon and prostate tumors as well as in oral squamous cell carcinomas. On the other hand, Brk expression has been correlated with differentiation in keratinocytes and Brk family kinases have been shown to have an inhibitory effect on receptor pathway signaling. To delineate the seemingly controversial role of this novel intracellular tyrosine kinase in proliferation and differentiation, mice carrying a disruption in the *brk* gene were generated. Since Brk is expressed at highest levels in the gastrointestinal tract, the continuously regenerating intestinal epithelium has been selected as a model system to analyze Brk signaling. In contrast to Src tyrosine kinases, Brk expression is restricted to differentiating cells of the rapidly renewing intestinal epithelium suggesting a role in the regulation of differentiation. The present study is focused on gaining a better understanding of the biological function of Brk in the gastrointestinal tract and its role in the development of cancer.

2 Materials and Methods

2.1. Mouse strains

Mice carrying a loss-of-function mutation of the *brk* gene were previously generated by Dr. Valeri Vasioukhin. The targeting construct contained the neomycin phosphotransferase (*neo*) gene, which was introduced into exon 1 in order to disrupt the initiation codon of the *brk* gene. In addition, the targeting construct included a diphtheria toxin A-fragment (DT) gene, which was introduced into the 3' end and used for negative selection that enriches homologous recombinants in embryonic stem (ES) cells (Yagi, et al., 1990). When non-homologous recombination occurred, the DT gene was expressed causing the death of these recombinants. Homologous recombination resulted in loss of the DT gene and the recombinant cells survived selection with G418 due to expression of the *neo* gene. The ES cells, derived from mouse strain 129, were screened for homologous recombination by PCR and confirmed by Southern Blotting. The cells were injected into 4-day embryos from C57BL/6 mice and embryos were fostered in pseudopregnant females. Chimeric animals were identified by coat color and males were mated with Swiss black females. Heterozygous animals were mated to generate four *brk* knockout lines designated 2.8, 32.7, 33.7 and 11.6. To produce a congenic strain deficient for the *brk* gene, knockout mice from the line 2.8 were backcrossed to C57BL/6 mice for 12 generations. This inbred mouse line was designated 2.8 G12. Mutant mice were identified by PCR and confirmed by Southern Blotting and RNase protection assays.

2.2. Mouse experiments

2.2.1. Induction of colitis using dextran sodium sulfate

Adult male wild-type and knockout mice ($n \geq 5$) were age-matched (age ≥ 7 weeks) and colitis was induced by feeding 3% dextran sodium sulfate (ICN), MW=40,000, dissolved in the drinking water *ad libitum* (Tessner, et al., 1998). 3% DSS was administered for 5 days followed by a recovery period with water for 3 days. Mice were studied at the end of 5 days of DSS treatment (5 days) and at the end of 3 days water after DSS (8 days) (Fig. 12A). Mice were monitored for rectal bleeding and weight loss for the duration of the study. The control group received

distilled water without DSS. At the time of sacrifice, the distal ileum, cecum and entire colon were excised and processed for biochemical and immunohistochemical analysis (see below).

2.2.2. Treatment of mice with AOM and DSS in a colon carcinogenesis model

Age- and sex-matched wild-type and knockout mice (n ≥ 20) were subjected to treatment with azoxymethane (AOM) in combination with dextran sodium sulfate (DSS) in a colon carcinogenesis model (Tanaka, et al., 2003). Mice were given a single intraperitoneal injection of AOM dissolved in saline (10 μg/ g bodyweight). Starting 1 week after the injection, animals received 2% DSS in the drinking water for 7 days and then no further treatment for 18 weeks (Fig. 14A). Animals were weighed weekly and monitored for rectal bleeding. At the end of the study (week 20), mice were sacrificed and their entire colon was excised and flushed with cold PBS. The colons were cut open longitudinally and washed with PBS. The tissue was fixed in 70% ethanol and macroscopically inspected.

2.2.3. γ-irradiation of wild-type and Brk knockout mice

Adult male wild-type and knockout mice (n ≥ 4) were age-matched (age ≥ 7 weeks) and exposed to ionizing radiation in a JL Shepherd Model 6810 ^{137}Caesium γ-irradiator (JL Shepherd). The animals were whole-body γ-irradiated at a dose of 8 Gγ on a rotating platform. The mice were sacrificed at 6 and 72 hours post irradiation representing the peak of early apoptosis and the point of crypt regeneration in the small intestine respectively (Potten, 1997, Potten, 1998 #3408). The distal jejunum and ileum were excised as described below. The tissues were processed for biochemical and histological analysis.

2.3. Tissue preparation and histology

Wild-type and knockout mice were sacrificed by CO_2 anesthesia followed by cervical dislocation. The gastrointestinal tract, including the entire small and large intestine, was removed and flushed with cold PBS with a syringe and large-bore needle. The small intestine was divided into pieces of tissue representing the proximal

duodenum (first 3 cm at proximal end), distal ileum (last 3 cm at distal end) and distal jejunum (3 cm distal from the center), and fixed in 4% paraformaldehyde in PBS overnight at 4°C. Specimens were dehydrated for embedding by passage through 75% ethanol for 45 minutes, 85% ethanol for 45 minutes, 95% ethanol for 45 minutes, 100% ethanol for 45 minutes (two times), xylene for 30 minutes (two times), and liquid paraffin embedding medium (Fisher) at 62°C for 1 hour (two times). Tissues were then mounted in paraffin blocks and sections of 5 µm thickness were made using a microtome. After drying, these paraffin sections were heated for 2 hours at 65°C to promote tissue adherence to the slides.

For the quantitative examination of villus height and cryptal depth, control and mutant adult mice ($n \geq 3$) were age-matched (7-10 weeks). Paraffin-embedded cross sections of distal ileum or jejunum were stained with hematoxylin and eosin. Samples were examined by light microscopy and images of the sections were captured. The measurements of villus height and cryptal depth were made for each animal using Adobe Photoshop 7.0 image analysis software. For each animal, 6 well-oriented villi and crypts from at least 5 quadrants (for a total of at least 30) of each cross section were measured. The average of each animal was used to determine an average villus height and cryptal depth for wild-type and knockout mice. The data gathered for each parameter assessed were compared by subjecting the results to Student's two-tailed t-test to determine the P-values.

2.4. Immunohistochemical techniques

Proliferation was detected by the incorporation of BrdU into S-phase cells during a 1-2 hours pulse just before death. Mice were injected intraperitoneally with 5-bromo-2-deoxyuridine (BrdU, Sigma) in PBS at 50 µg/ g bodyweight. Gastrointestinal tissues were dissected as described above and fixed in Carnoy's (10% glacial acetic acid, 60% ethanol, 30% chloroform) solution for 3 hours at 4°C. Tissues were then washed and processed for embedding as described above. BrdU immunohistochemistry was performed using a M.O.M. mouse on mouse Immunodetection Kit (Vector) following manufacturer's instructions. Briefly, sections were deparaffinized in xylene two times for 5 minutes and rehydrated by sequential passage through 100%, 100%, 70%, 50%, and 30% ethanol for 3 minutes

each. After washing in PBS, antigen retrieval was performed in antigen demasking solution (Vector) for 20 minutes at 90°C. After washing in TNT (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween 20), sections were treated with 1 µg/ml Proteinase K for 10 minutes followed by incubation with 3% H_2O_2 in methanol for 10 minutes and incubation with 70 mM NaOH for 3 minutes. Samples were blocked using an Avidin/Biotin blocking Kit (Vector) according to manufacturer's instructions followed by incubation with M.O.M. mouse IgG blocking reagent for 1 hour at room temperature. The blocking reagent was removed and slides were pretreated with M.O.M. diluent (Vector) for 5 minutes before incubation with anti-BrdU monoclonal antibody (Becton Dickinson) 1:75 in M.O.M. diluent at room temperature for 1 hour. Slides were washed in TNT buffer three times for 5 minutes and incubated with M.O.M. biotinylated anti-mouse IgG reagent (Vector) for 30 minutes at room temperature. The BrdU-signal was visualized by incubation of the slides with avidin-horseradish peroxidase (Vector) for 30 minutes followed by incubation with the chromogenic substrate 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) for 10 minutes. Nuclei were counterstained with hematoxylin. The sections were dehydrated by sequential passage through 30%, 50%, 70%, 100%, and 100% ethanol, then passed through xylene and mounted with cover slips and Permount (Vector).

For further immunohistochemical analyses, tissue sections were processed using the Vectastain ABC Kit (Vector) according to manufacturer's instructions. Briefly, after deparaffinizing and rehydrating the 5 µm tissue sections, the slides were washed in TNT and antigen retrieval was performed by microwaving the samples in 10 mM sodium-citrate buffer ($C_6H_7O_7Na$) pH 6.0 four times for 5 minutes each, replacing evaporated buffer as needed. To quench endogenous peroxidase activity, slides were incubated with 3% H_2O_2 in methanol for 10 minutes followed by washing in TNT for 5 minutes. Samples were then blocked in goat or horse serum in PBS (Vector) for 30-60 minutes at room temperature. Tissues were incubated in blocking buffer with the primary antibody for 1 hour at room temperature for anti-Brk polyclonal antibody (Santa Cruz) 1:50, anti-PCNA polyclonal antibody (Santa Cruz) 1:200, anti-β-catenin monoclonal antibody (BD Biosciences) 1:200, and overnight at 4°C for anti-c-myc polyclonal antibody (Santa Cruz). Controls were performed with equal dilutions of rabbit or mouse IgG (Santa Cruz). Detection of the primary antibody was performed by incubation with goat anti-rabbit or horse anti-mouse

biotinylated secondary antibody (Vector) for 30 minutes followed by incubation with avidin-horseradish peroxidase (Vector) for 30 minutes according to the Vectastain ABC Kit (Vector). To visualize the signal, sections were incubated in DAB (Sigma) for 5-10 minutes at room temperature. Reactions were stopped by washing in water for 10 minutes. Counterstaining with hematoxylin was performed as indicated. The sections were dehydrated and mounted as described above.

For immunohistochemical analysis of tight junctions, tissue sections were stained with anti-ZO-1 monoclonal antibody conjugated to FITC (Zymed laboratories). Briefly, after deparaffinizing and rehydrating, slides were washed in TNT and antigen retrieval was performed by microwaving the samples in 10 mM sodium-citrate buffer ($C_6H_7O_7Na$) as described above. Samples were then blocked in horse serum in PBS (Vector) for 60 minutes at room temperature. Tissues were incubated with the primary antibody anti-ZO-1-FITC 1:200 in 1% BSA in PBS overnight at 4°C. Nuclei were counterstained with 2 µg/ml 4',6-diamidino-2-phenylindole (DAPI, Sigma). Sections were coverslipped with Vectashield mounting medium for fluorescence (Vector) and examined by fluorescence microscopy.

To detect cleavage of caspase-3 in the gastrointestinal tract, tyramide signal amplification was performed. Sections were deparaffinized and rehydrated as described above. Antigen retrieval was performed by boiling sections in 1mM EDTA pH 8.0 for four times 5 minutes each. Samples were blocked with TNB buffer (NEN, 0.1 M Tris, pH7.5, 0.15 M NaCl, 0.5% Blocking Reagent) for 1 hour at room temperature and incubated with anti-cleaved-Caspase-3 polyclonal antibody (Cell Signaling) 1:100 in TNB overnight at 4°C. As a control, sections were incubated with rabbit IgG (Santa Cruz) 1:100. After washing and incubation with biotinylated goat anti-rabbit antibody (Vector) for 60 minutes, the TSA-indirect kit (NEN) was used according to manufacturer's instructions. Briefly, sections were treated with streptavidin-horseradish peroxidase (1:100, NEN), reacted for 8 minutes with biotinylated tyramide reagent (NEN), and visualized with FITC-Avidin DCS (Vector). Nuclei were counterstained with DAPI (Sigma). Sections were coverslipped with Vectashield mounting medium for fluorescence (Vector) and examined by fluorescence microscopy. The number of cleaved Caspase 3-positive cells per crypt-villus unit was scored for each animal. For quantification, positive cells were counted for at least 10 crypt-villus units from at least 5 quadrants (a minimum of total 50) for

each animal and used to determine the average of each animal. The data gathered were compared by subjecting the results to Student's two-tailed t-test to determine the P-values.

2.5. Expression constructs, cell culture and stable cell lines

Wild-type Brk (Brk WT) and Brk Y-F coding sequences were cloned into the retroviral expression vector pLXSN (Vasioukhin and Tyner, 1997). Brk Y-F has a substitution of the regulatory tyrosine at position 447 of wild-type mouse Brk to phenylalanine, resulting in a constitutively activated mutant of the kinase. Phoenix cells and Rat1A fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with penicillin (100 U/ml) (Invitrogen), streptomycin (100 µg/ml) (Invitrogen) and 10% fetal bovine serum (Atlanta Biologicals) at 37°C in 5% CO_2.

Retroviral transduction was performed using the Phoenix ecotrophic packaging cell line (Kennedy, et al., 1997). Phoenix cells were plated at approximately 50-75% confluence in 15-cm-diameter tissue culture plates and transfected with the control neomycin vector pLXSN, wild-type Brk and activated Brk Y-F constructs by calcium phosphate co-precipitation in the presence of 0.2 M chloroquine (Pear, et al., 1993, Honigwachs, 1989 #3500). Cells were washed after 24 hours, and the high-titer retroviral supernatants were collected at 48 and 72 hours post transfection. Retroviral infection of Rat1A fibroblasts was performed by incubation of a 50% confluent 10-cm dish with 1.5 ml of retrovirus-containing supernatant in the presence of 8 µg/ml Polybrene (Fisher) for 24 hours. After the incubation, retrovirus expressing cells were selected with 300 µg/ml G418 (Invitrogen). The cells were pooled, expanded, and maintained as polyclonal populations. After establishment, cell lines were frozen and stored in liquid nitrogen, and a fresh aliquot of cells was used for each set of experiments.

2.6. Cell treatments

Polyclonal Rat1a fibroblasts stably expressing wild-type (Brk WT) and activated Brk (Brk Y-F), and the control neomycin vector pLXSN, were generated as described above. For growth rate determinations, 1 x 10^5 cells seeded in 60 mm dishes were fed and counted daily for 5 days using a Hematocytometer. Three plates were counted for each time point and cell line to determine the average cell number.

For apoptotic assays, the cells were plated on 6-well culture dishes at 1 x 10^5 cells/ well 16 hours before treatment. Cells were then either serum starved or subjected to a combination of serum starvation and UV-irradiation. For serum starvation experiments, cells were grown in 0% FBS for 24 hours. For UV-irradiation experiments, cells were placed in PBS and irradiated with UV light (50 J/m^2) (Kennedy, et al., 1999). After UV-irradiation, PBS was replaced with DMEM containing no serum and cells were incubated for 3, 3.5, 4 and 4.5 hours at 37°C in 5% CO_2. After the indicated treatment, cells were fixed by the addition of formaldehyde at a final concentration of 18.5% and incubated for 24 hours at room temperature. Cells were then rinsed with PBS and stained with 2 μg/ml DAPI for 3 minutes. The percentage of apoptotic cells was visualized under an inverted fluorescence microscope. Fragmented and condensed nuclei were scored as apoptotic with at least 200 cells per experimental group. All treatments were done at least in triplicate, and the data for each experiment was used to determine the average amount of cell death. Results were subjected to Student's two-tailed t-test to determine the P-values.

2.7. Flow cytometry

For cell cycle analysis, combined detection of BrdU incorporation and DNA content using propidium iodide (PI) was performed, using CellQuest software on a FACSCalibur flow cytometer (Becton Dickinson) and a BrdU flow kit (BD Biosciences) according to the manufacturer's recommendations. Polyclonal Rat1A fibroblasts stably overexpressing the empty expression vector pLXSN (control), wild-type Brk (Brk WT) and the activated mutant of Brk (Brk Y-F) were synchronized in DMEM containing 0.1% FBS for 48 hours. The medium was replaced with DMEM containing 10% FBS, and after the indicated time-points (0, 2, 4, 8 and 16 hours) the

cells were pulse labeled with 100 µM BrdU (Sigma) for 40 min. Cells were harvested by trypsinizing, pelleted by centrifugation for 5 min at 1200 rpm, and 1 x 10^6 cells were resuspended in 0.5 ml ice cold PBS. Cells were then fixed by drop-wise addition to 70% ice-cold Ethanol and stored overnight at 4°C. Subsequently, cells were pelleted by centrifugation, washed in PBS containing 0.5% BSA and denatured in 2N HCl/0.5% Triton X-100 solution for 20 minutes at room temperature. The cells were washed in PBS/0.5% BSA and residual acid was neutralized by incubation of the cell pellet with 0.1M sodium borate, pH 8.5 for 2 minutes at room temperature. After an additional wash cells were immunostained with 0.5 µg FITC-conjugated mouse anti-BrdU monoclonal antibody (BD Biosciences) for 30 minutes at room temperature, protected from light, and centrifuged after addition of 1 ml of PBS/0.5% BSA. The cell pellet was then resuspended in 0.5 ml staining solution containing 20 µg/ml of propidium iodide (Molecular Probes) and 200 µg/ml of RNase A (Sigma) in 0.1% Triton X-100. After at least 30 minutes of incubation at room temperature, the cell suspension was passed through a 35-µm-pore-size cell strainer (Fisher) and analyzed by two-parameter flow cytometry using a Becton Dickinson FACSCalibur flow cytometer.

For analysis of apoptosis, serum starved or irradiated/starved stable Rat1A populations (treatments as above) expressing vector only (control), Brk WT and Brk Y-F were subjected to FACS analysis. After collection of floating cells and trypsinization of attached cells, the pooled fractions (1 x 10^6) were rinsed with PBS, resuspended in 0.5 ml of PBS and fixed by drop-wise addition to ice-cold 70% ethanol and stored for at least 16 hours at 4°C. Subsequently, cells were pelleted by centrifugation and incubated in 0.5 ml staining solution containing 20 µg/ml of propidium iodide (Molecular Probes) and 200 µg/ml of RNase A (Sigma) in 0.1% Triton X-100 for 30 min at room temperature. The cell suspension was passed through a 35-µm-pore-size cell strainer (Fisher) and flow cytometry was performed using a Beckton Dickinson FACSCalibur flow cytometer. To determine the cell cycle distribution by propidium iodide staining, data collected from the FACsort was analyzed using CellQuest and ModFit software (Becton Dickinson). For the purpose of analysis, acquired events were gated to eliminate cell aggregates and debris. A gated population of single diploid cells was analyzed.

2.8. Ribonuclease protection assays

Total RNA was prepared after the guanidine iso-thiocyanate method (Chomczynski and Sacchi, 1987) using TRIzol reagent (Invitrogen). Mouse tissues were dissected quickly and immediately homogenized in 3 ml TRIzol reagent. Total RNA was isolated following the manufacturer's instructions.

Brk expression in the gastrointestinal tract was analyzed as described previously (Siyanova, et al., 1994), using [^{32}P]-CTP-labeled antisense RNA probes. A pBlueScript SK II+ plasmid containing a 205-bp fragment encoding a portion of the mouse Brk catalytic domain was linearized at an XbaI site in the polylinker. As control for RNA levels and integrity, a pTRI plasmid (Ambion) containing a fragment of mouse cyclophilin was linearized. *In vitro* transcription was performed using T7 polymerase (Promega).

Expression of various cytokines in the murine gastrointestinal tract was analyzed by RNase protection assay using the RiboQuant multiprobe RNase protection assay system (BD Biosciences) according to the manufacturer's instructions. Briefly, the mCK-2b multiprobe DNA templates were used for synthesis of [^{32}P]-α-UTP-labeled anti-sense RNA probes by T7 polymerase-directed *in vitro* transcription. The highly-specific RNA probes can hybridize with target mouse mRNAs encoding interleukin-12p35 (IL-12p35), IL-12p40, IL-10, IL-1α, IL-1β, IL-1Ra, IL-18, IL-6, IFN-γ, migration inhibition factor (MIF) as well as housekeeping genes L32 and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) for assessment of RNA levels.

Twenty µg of total RNA for each sample or of yeast tRNA were precipitated with ethanol and resuspended in 30 µl hybridization buffer containing 3.95 x 10^5 cpm/µl of probe. Samples were hybridized for 12-16 hours at 56°C followed by treatment with 100 µl of ribonuclease digestion buffer containing 192 x 10^{-3} ng/ml of RNase A and 0.6 units/µl RNase T (BD Biosciences) for 45 minutes at 30°C. Samples were subjected to proteinase K digestion, ethanol precipitation, and subsequently resolved on denaturing polyacrylamide gels as described. The gel was dried and protected fragments were visualized by exposing to X-ray film (Midwest Scientific).

2.9. Protein lysates and immunoblotting

Mouse tissues were dissected as described above and immediately homogenized in 1.5 ml of cold lysis buffer (20 mM Hepes, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, pH 8.0, 1 mM EGTA, pH8.0, 10 mM Na-pyrophosphate, 100 mM NaF, 5 mM Iodoacetic Acid, 0.2 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A) using a polytron (Kinematica AG) at 5,000 rpm. After incubation on ice for 20 minutes, samples were sonicated for 15 seconds and centrifuged for 30 minutes at 30,000 rpm at 4°C.

Cells were grown as previously described. For total cell lysates, the cells were washed with ice-cold PBS and incubated in ice-cold lysis buffer (see above) for 20 minutes on ice with constant agitation. The cells were harvested with a cell scraper (Fisher Scientific), and the cell extracts were cleared by centrifugation at 14,000 rpm for 10 minutes.

Thirty micrograms of total cell lysate or 50-100 µg of tissue lysate were resuspended in SDS gel-loading buffer (0.15 M Tris-Cl, pH 6.8, 15% SDS, 50% glycerol, 1.5% bromphenol blue, 15% β-mercaptoethanol). Samples were boiled for 5 minutes and separated by SDS-PAGE at 60 V (0.4 V/cm^2) through the separating and 120 V (0.7 V/cm^2) through the resolving gel. The proteins from the SDS-polyacrylamide gels were transferred to polyvinylidene-difluoride (PVDF) membranes (Immobilon), as described (Sambrook, et al., 1989). Transfer was performed in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol) overnight at 20 V at 4 °C.

For detection of proteins, the membranes were incubated in blocking solution (5% nonfat dry milk in TBS-T (10 mM Tris-HCl, pH 7.5, 100 mM NaCl or 200 mM NaCl for Brk, 0.1% Tween 20) for 1 hour at room temperature. The filters were then incubated with primary antibody for 1 hour at room temperature (anti-mouse Brk polyclonal antibody 1:2000 and anti-Erk1/2 polyclonal antibody 1:2000 (Santa Cruz), anti-β-actin monoclonal antibody 1:5000 (Sigma)) or overnight at 4°C (anti-Phospho-Erk1/2 polyclonal antibody 1:1000, anti-Phospho-Ser473-Akt polyclonal antibody 1:1000, anti-Akt polyclonal antibody 1:1000, anti-Phospho-GSK-3α/β(Ser21/9) polyclonal antibody 1:1000, anti-GSK-3β polyclonal antibody 1:1000 (Cell Signaling)). Primary antibodies were detected using horseradish peroxidase-

conjugated donkey anti-rabbit or sheep anti-mouse secondary antibodies (Amersham) at a dilution of 1:5000 for 1 hour at room temperature. Peroxidase reaction was visualized with SuperSignal West Dura Extended Duration Substrate for chemiluminescence (Pierce) and membranes were exposed to X-ray film.

2.10. Immunoprecipitations

Three hundred to 500 µg of tissue lysate were used per immunoprecipitation reaction. The volume of each sample was adjusted to 1 ml with lysis buffer and the samples were precleared by incubation with 1 µg of rabbit IgG (Santa Cruz) and 75 µl of 50% Protein A sepharose slurry (Amersham) for 1 hour at 4°C. After centrifugation for 10 minutes at 13,000 rpm at 4°C, the supernatant was transferred to a fresh eppendorf tube and incubated with 1 µg of anti-Akt polyclonal antibodies (Cell Signaling) overnight at 4°C on a rotary shaker. As controls, the lysate was incubated with 1 µg of rabbit IgG (Santa Cruz). The next day, 50 µl of 50% Protein A sepharose slurry were added to the lysate and incubated for 1 hour at 4°C, rotating. After incubation, immune complexes were collected by centrifugation for 10 minutes at 13,000 rpm at 4°C. The supernatant was aspirated and the beads were washed three times in lysis buffer. After the last wash, the beads were resuspended in 20 µl 2x sample buffer and the proteins denatured by boiling for 5 minutes. Proteins were resolved by 9% SDS-PAGE, transferred to membranes, and analyzed by Western blotting with the indicated antibodies.

2.11. *In vitro* Akt kinase assays

In vitro Akt kinase assays were performed using a Nonradioactive Akt Kinase Assay Kit (Cell Signaling) according to the manufacturer's instructions. Briefly, 300-500 µg of tissue lysates were incubated with immobilized Akt (1G1) monoclonal antibody overnight at 4°C. The resulting immune complexes were washed two times in cell lysis buffer and two times in kinase buffer. Then, *in vitro* kinase assay was performed using recombinant GSK-3 fusion protein as a substrate. The Akt immunoprecipitates were incubated with 0.2 mM ATP and 1 µg GSK-3 fusion protein in kinase buffer for 30 minutes at 30°C. Reactions were terminated with 25 µl 3x

sample buffer, boiled for 5 minutes and the supernatant was separated by 12% SDS-PAGE. Phosphorylation of GSK-3 was measured by Western blotting using Phospho-GSK-3α/β (Ser21/9) polyclonal antibody.

3 Results

3.1. Brk is required for intestinal homeostasis

3.1.1. Brk protein expression is restricted to differentiated cells

Expression of the gene encoding the non-receptor tyrosine kinase Brk is restricted to epithelial cells of the skin, prostate, and gastrointestinal tract in the mouse (Derry, et al., 2003; Llor, et al., 1999; Vasioukhin, et al., 1995). Brk mRNA expression is developmentally regulated. It is detected late in gestation in the mouse as regenerating epithelia begin to mature. Furthermore, mRNA expression is restricted to non-proliferating epithelial cells, suggesting a biological function for Brk in the process of differentiation in these epithelial tissues (Vasioukhin, et al., 1995).

To confirm the developmentally regulated expression pattern of Brk based on mRNA studies, Brk protein expression throughout the gastrointestinal tract of wild-type mice was examined by immunohistochemistry (Fig. 4). Jejunum and colon of adult mice were extracted, fixed in 4% paraformaldehyde, embedded and 5 µm paraffin sections were incubated with anti-Brk antibodies or anti-IgG antibodies as controls. Reactions were detected with the Vector ABC Kit and visualized with DAB. Nuclei were counterstained with hematoxylin. Brk protein was detected in both jejunum and colon, where its expression was restricted to epithelial cells that exit the cell cycle and undergo terminal differentiation (Fig. 4). This highly specific expression pattern supported the hypothesis that the Brk tyrosine kinase plays a role in promoting differentiation in normal regenerating epithelial linings.

To investigate a putative function for the non-receptor tyrosine kinase Brk in the differentiation process of the gastrointestinal tract, mice deficient for Brk were generated (Wenjun Bie, 2005). No overt phenotypic changes were observed in Brk deficient mice. They were viable, fertile and did not develop spontaneous tumors (Wenjun Bie, 2005). These findings suggested that a high degree of redundancy exists between different members of the Brk family.

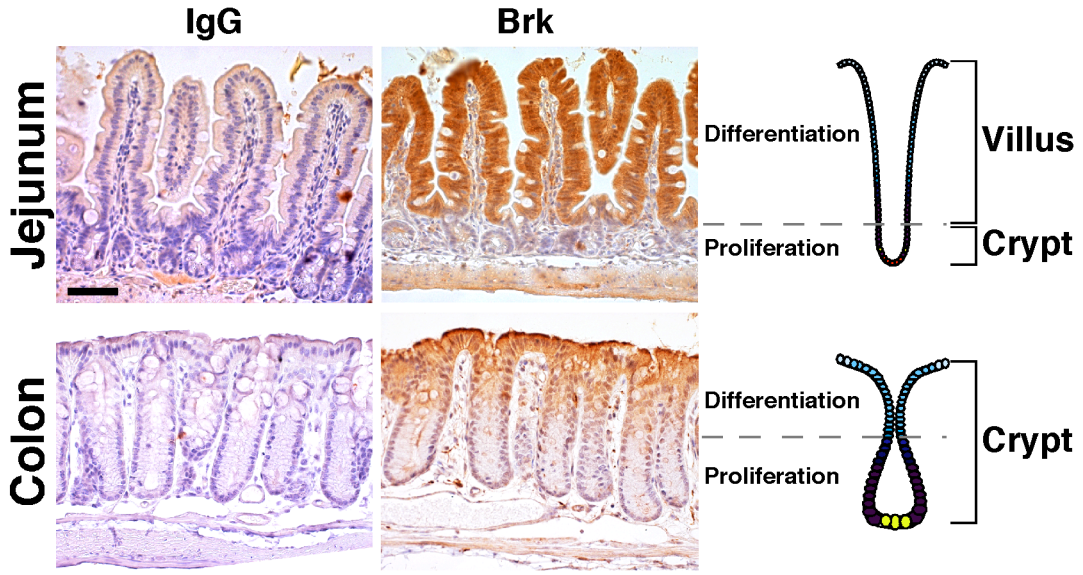

Fig. 4: Expression of Brk protein in the gastrointestinal tract. Immunohistochemistry for Brk in adult wild-type jejunum and colon using anti-Brk antibodies or anti-rabbit IgG antibodies as controls and DAB as a substrate (brown). Nuclei were counterstained with hematoxylin (blue). Size bar represents 50 μm. Brk expression is restricted to differentiated epithelial cells.

Loss of Brk mRNA expression in mutant mice was confirmed by RNase protection assay (Fig. 5A). Furthermore, expression of Brk protein was compared in neonatal and adult ileum and colon by Western blotting (Fig. 5B). Tissue lysates were separated by SDS-page and immunoblotting for Brk and β-actin as a loading control were performed. Highest levels of Brk protein were detected in neonatal colon, whereas adult mice showed peak levels of Brk protein in ileum (Fig. 5B). These data suggested that Brk protein expression is regulated along the length of the intestine. Differential regulation during development has also been shown for Brk mRNA expression (Llor, et al., 1999). No Brk protein expression was observed in the intestine of knockout mice, confirming the loss-of-function mutation in the Brk gene (Fig. 5B).

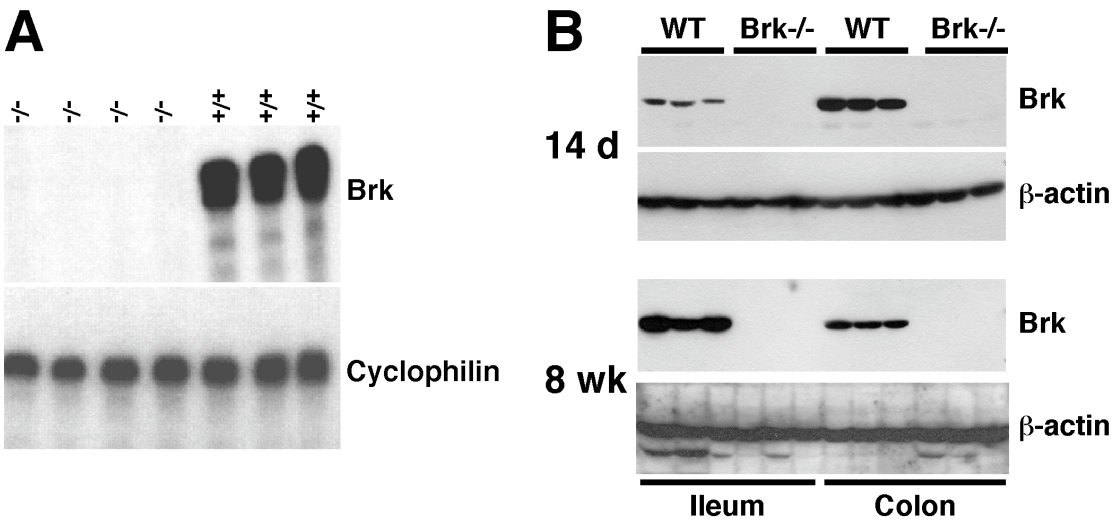

Fig. 5: Brk mRNA and protein expression in the gastrointestinal tract. (A) RNase protection analysis for Brk mRNA synthesis in homozygous mutant and wild-type mice. The loading control was performed with a probe for cyclophilin. (B) Western blot analysis for Brk synthesis in neonatal (14 days) and adult (8 weeks) wild-type (WT) and mutant (Brk -/-) ileum and colon. The loading control was performed with anti-β-actin antibodies.

3.1.2. Increased epithelial cell turnover in Brk knockout mice

To investigate the influence of Brk on the homeostatic balance of the gastrointestinal epithelium, wild-type and knockout mice were examined for their proliferative state. Age-matched mice were pulse labeled with BrdU 2 hours prior to sacrificing, and their gastrointestinal tract was dissected, fixed and embedded. Paraffin sections of the small intestine and colon were subjected to immunohistochemistry with antibodies directed against PCNA and BrdU (Fig. 6A, B). Analysis of PCNA- and BrdU-positive intestinal epithelial cells revealed an increased number of proliferating cells in Brk knockout mice compared to wild-type controls (Fig. 6A, B). Importantly, the proliferating cells were present in the middle and upper regions of the crypt in Brk knockout mice. These areas of the crypt are remote from the stem cell area and are normally fully differentiated and non-proliferating. The expanded proliferative zone and increased number of proliferating cells in Brk deficient mice suggested a deregulated balance between proliferation and differentiation in the intestinal epithelium in the absence of Brk signaling.

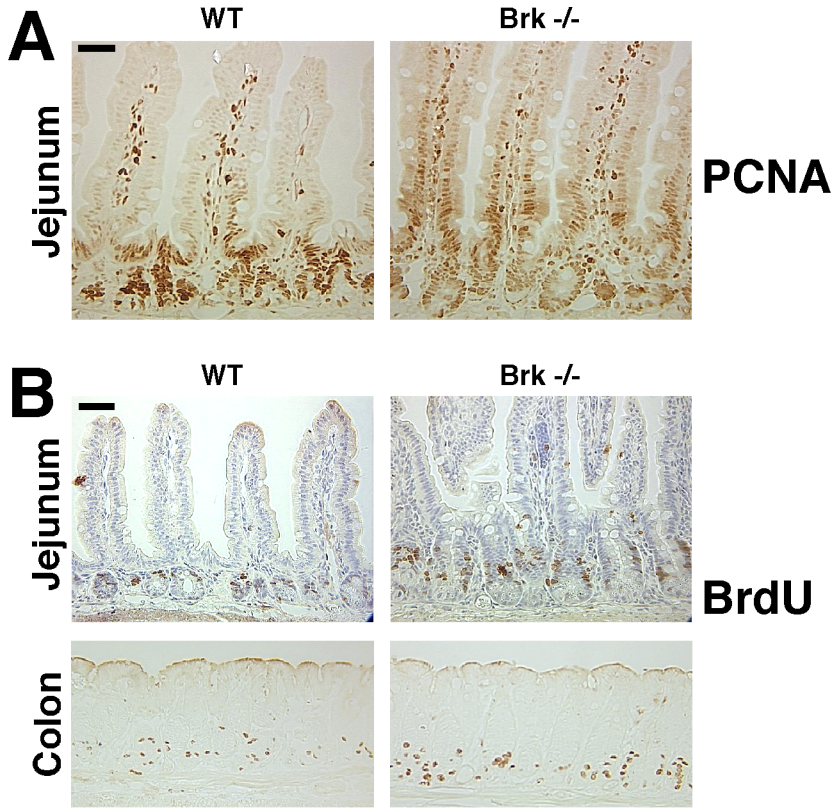

Fig. 6: Defects in intestinal epithelial homeostasis in the absence of Brk signaling. Photomicrographs of immunohistochemical staining for PCNA (A) and BrdU (B) (brown) from sections of jejunum and colon of wild-type (WT) and knockout (Brk -/-) mice. Mice were injected with 50 *µ*g BrdU/ g bodyweight and sacrificed 2 hours later. Sections were counterstained with hematoxylin (blue). Size bars represent 50 *µ*m. The amount of proliferation indicated by PCNA and BrdU positive cells is increased in the mutant epithelium.

In addition, paraffin sections of the distal ileum and jejunum of age-matched wild-type and knockout mice were stained with hematoxylin and eosin and observed by light microscopy (Fig. 7A, C). Cross visual comparison between wild-type and knockout mice showed an increased height of intestinal villi in knockout mice. To further examine and quantify this difference, villus height and crypt depths were measured for at least 3 animals per group and tissue with at least 30 scores each (Fig. 7B, D). The resulting data showed a statistically significant difference (P-value ≤ 0.05) in villus height of the distal ileum and distal jejunum of wild-type and knockout mice. The villi in Brk deficient mice were significantly longer than in their wild-type counterparts. On the other hand, no statistically significant difference was measured for the crypt depths of the analyzed tissues (P-value = 0.11) (data not shown). To ensure that the differences in villus height are not due to differences in general body size, total animal weight and length of the entire small intestine were measured. No

significant difference in either parameter was observed in wild-type and knockout mice (data not shown). In conclusion, these data suggested that the observed increase in proliferation in the intestinal epithelium of Brk knockout mice contributes to the phenotypic appearance of longer villi.

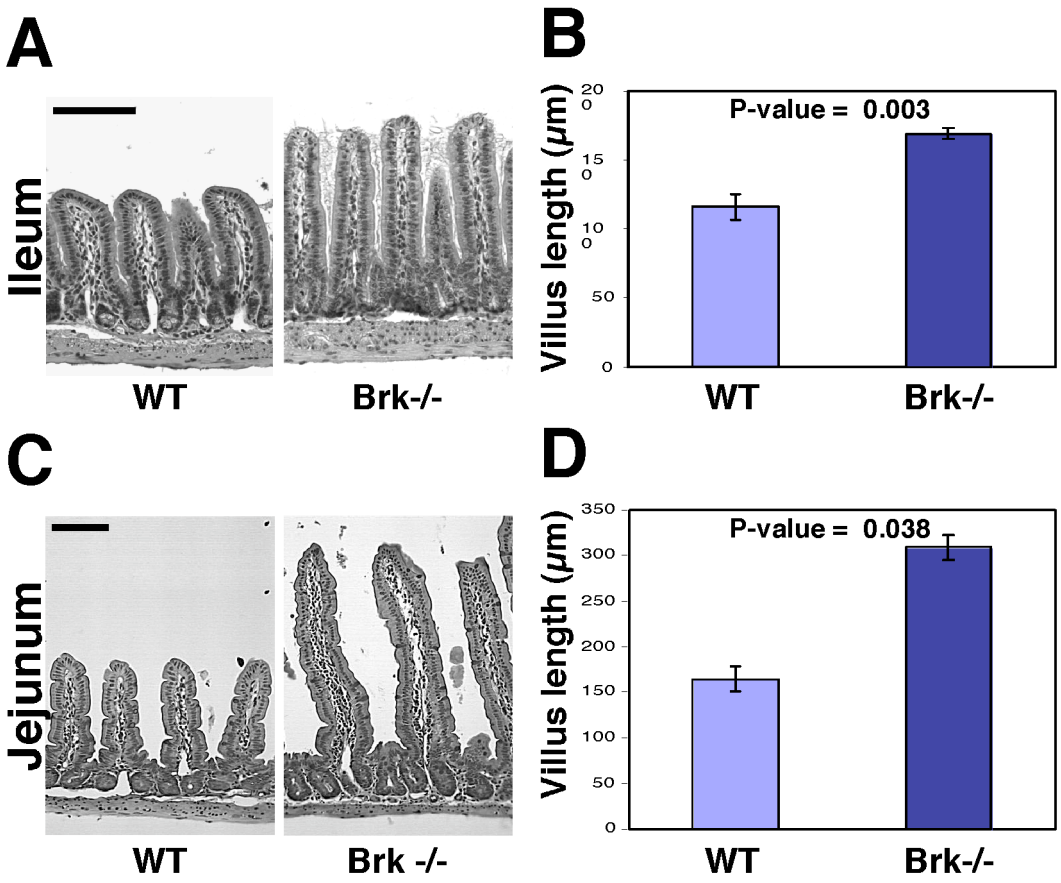

Fig. 7: Loss of Brk expression affects the crypt-villus morphology. (A, C) Representative sections of the distal ileum and distal jejunum from wild-type (WT) and knockout (Brk -/-) mice stained with hematoxylin/eosin. Size bars represent 100 µm. (B, D) Histograms of average villus length in wild-type and knockout distal ileum (B) and jejunum (D). Error bars represent ± SD. P-values were determined using the Student's test. The average villus length is drastically increased in the mutant mucosa.

3.1.3. Increased accumulation of nuclear β-catenin in the absence of Brk

The expansion of the progenitor zone in Brk deficient mice suggested that Wnt signaling might be affected in the intestines of these mice. Wnt signaling has been shown to define the intestinal epithelial progenitor cell phenotype (van de Wetering, et al., 2002). Activation of canonical Wnt signaling results in accumulation of nuclear β-catenin, which in complex with TCF-4 controls proliferation versus differentiation in intestinal epithelial cells (Batlle, et al., 2002; Pinto, et al., 2003). To determine by which molecular mechanism loss of Brk could affect proliferation in the adult intestine, the expression of β-catenin and its target gene *c-myc* was examined in the small and large intestine of wild-type and knockout mice. Immunostaining with anti-β-catenin antibodies revealed membrane-localized β-catenin along the crypt-villus axis as well as nuclear β-catenin in cells occupying basal positions of the crypt in sections of small and large intestine (Fig. 8A, B). Interestingly, comparing numerous fields, an increased number of cells positive for nuclear β-catenin were observed in Brk knockout mice compared to their wild-type counterparts (Fig. 8A-C).

To address, whether this Brk-dependent up-regulation of nuclear β-catenin affected the expression of β-catenin target genes, c-Myc expression in the gastrointestinal tract was analyzed. It has been previously shown that the formation of nuclear β-catenin/Tcf4 complexes results in the direct up-regulation of *c-myc*, which in turn represses $p21^{CIP1/WAF1}$ by direct promoter binding, thereby allowing cells to proliferate (He, et al., 1998; van de Wetering, et al., 2002). Distal jejunum and colon sections of wild-type and Brk knockout mice were immunostained with anti-c-Myc antibodies or anti-IgG as controls (Fig. 9A, B). In both wild-type and knockout animals, c-Myc protein expression was detected in the nucleus of crypt cells and c-Myc expression was absent from differentiated villus cells. However, knockout mice exhibited an increased c-Myc expression when compared to wild-type controls.

Taken together, these results demonstrated that in the absence of Brk signaling β-catenin and its target gene *c-myc* are induced. Loss of Brk expression activated the Wnt pathway, which might contribute to and/or result in the increased proliferation observed in Brk deficient mice. These *in vivo* findings supported a proposed role for Brk in maintaining intestinal epithelial cells in a non-proliferative, differentiated state. Mutations in the *APC* gene resulting in increased accumulation of nuclear β-catenin

have been shown to promote the development of spontaneous intestinal tumors, as shown in the case of *APC*^{MIN} mice (Moser, et al., 1992; Su, et al., 1992). Thus, loss of Brk and the resulting activation of nuclear β-catenin might sensitize these knockout mice to intestinal tumorigenesis.

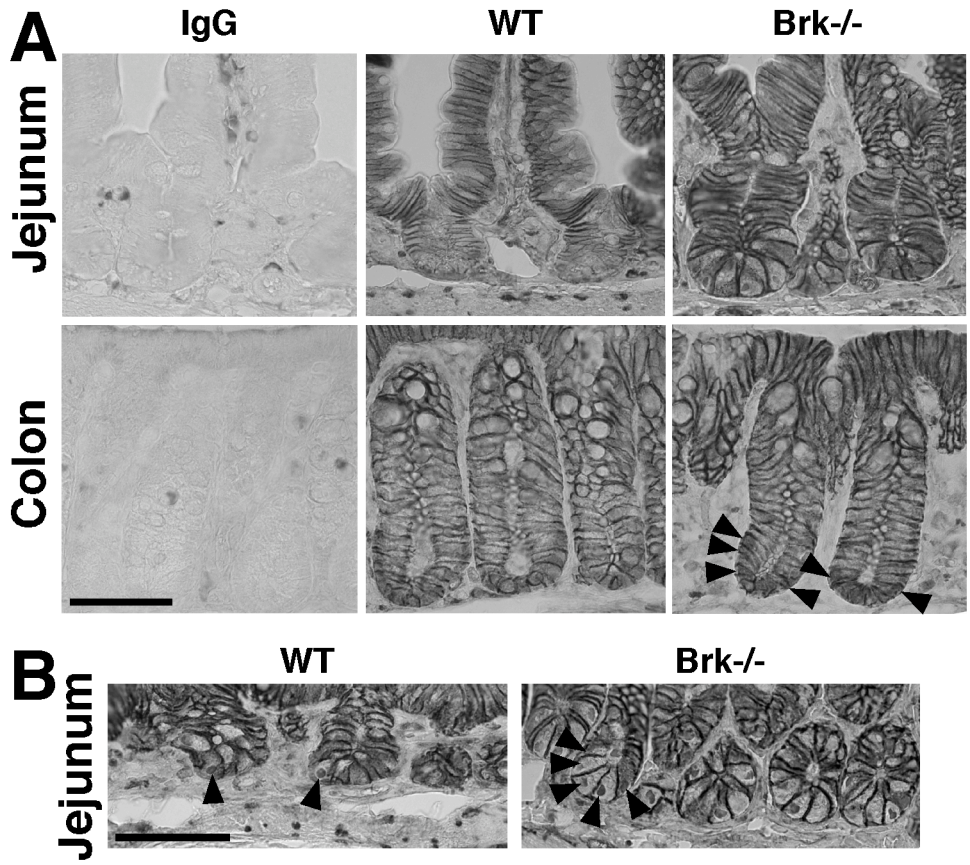

Fig. 8: Increased nuclear β-catenin expression in mutant intestinal epithelium. (A, B) Immunohistochemical analysis of wild-type (WT) and knockout (Brk -/-) distal jejunum and colon sections with antibodies against β-catenin and IgG as controls. Staining of β-catenin shows accumulation in the nucleus of cells at the crypt base (arrowheads), but is absent from the nucleus of cells along the crypt-villus axis, where it is only membrane-localized. Size bars represent 50 μm. Nuclear β-catenin is detected in more crypt cells of mutant intestinal epithelium compared to wild-type mice.

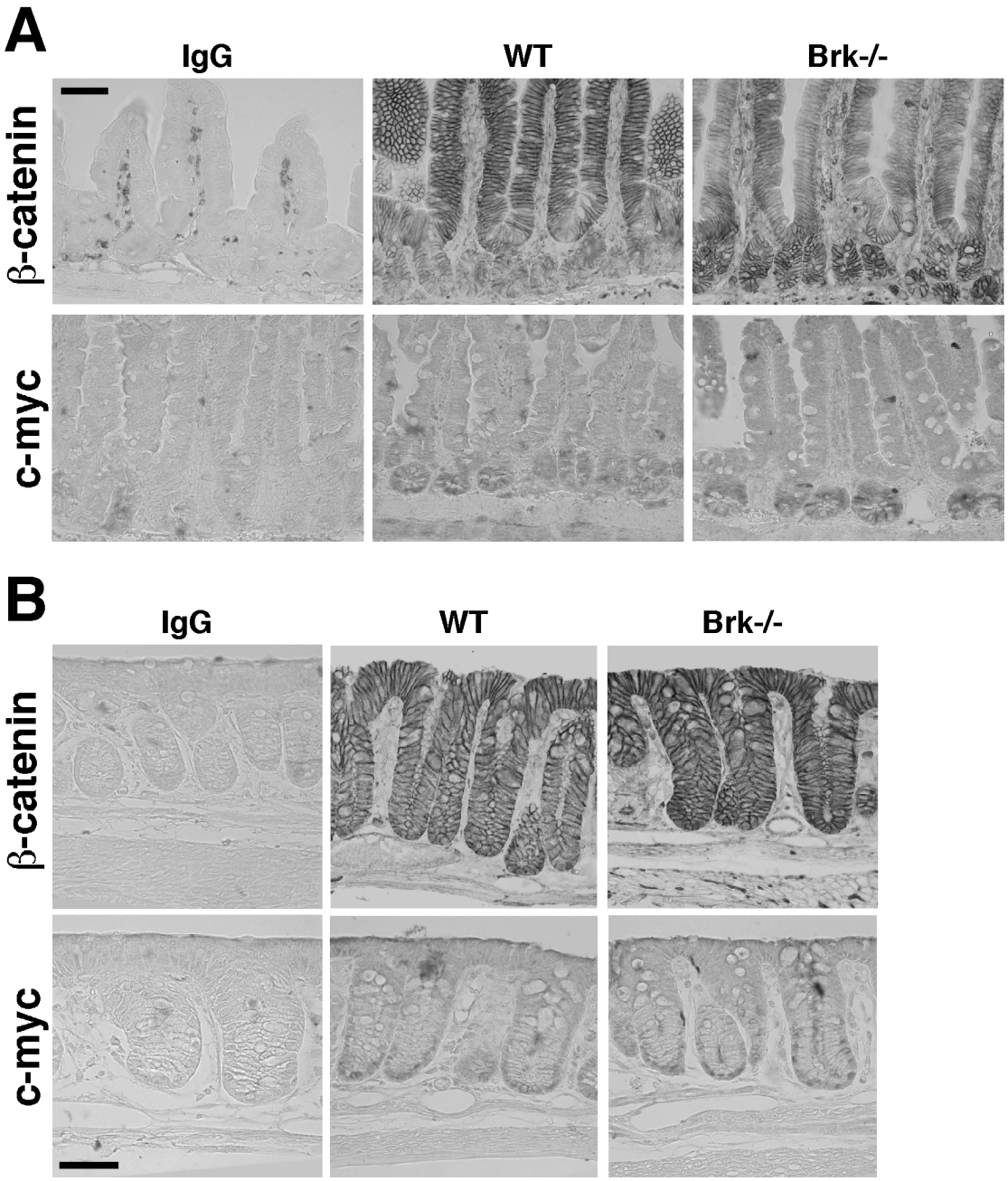

Fig. 9: Expression of nuclear β-catenin and its target gene *c-myc* in mutant intestinal epithelium. Representative sections of the distal jejunum (A) and colon (B) from wild-type (WT) and knockout (Brk -/-) adult animals were stained with anti-β-catenin and anti-c-myc antibodies. Immunostaining with anti-IgG antibodies served as controls. Size bars represent 50 *μ*m. Brk mutant mice exhibit increased nuclear β-catenin accumulation and upregulated expression of the β-catenin target gene *c-myc* in the intestinal crypts.

3.1.4. Regulation of β-catenin activity by Brk may be mediated by Akt

Multiple signaling pathways can impinge on β-catenin, affecting its stability and/or subcellular localization. It has been reported that Wnt signaling is required for the nuclear activity of β-catenin, but is not sufficient to fully activate it (He, et al., 2004). The survival kinase Akt has been shown to further facilitate the stabilization and nuclear accumulation of β-catenin, mainly acting through Dishevelled and GSK-3β but also through direct phosphorylation of β-catenin (Fukumoto, et al., 2001; Persad, et al., 2001; Tian, et al., 2004). A recent study showed that Brk binds to and inhibits Akt kinase signaling (Zhang, et al., 2004).

To study, whether Akt is involved in the regulation of β-catenin by Brk, tissue lysates from distal ileum of adult wild-type and knockout mice were analyzed for Akt kinase activity (Fig. 10). Endogenous Akt was immunoprecipitated using immobilized Akt antibodies and *in vitro* kinase assays were performed by incubation with ATP and purified recombinant GSK-3 protein. Samples were subjected to Western blotting using phospo-specific GSK-3 antibodies. The analysis showed that Akt activity was consistently increased in mice deficient for Brk compared to wild-type mice from either an outbred (2.8) or inbred (2.8 G12) background (Fig. 10). Interestingly, higher levels of basal Akt kinase activity were observed in inbred mice. These data suggested that Brk signaling might inhibit β-catenin activity through the Akt pathway. Brk signaling may therefore have a role in balancing the role of Wnt-β-catenin in promoting stem cell self-renewal (Reya, et al., 2003). Loss of Brk may facilitate the activation of β-catenin by the survival kinase Akt.

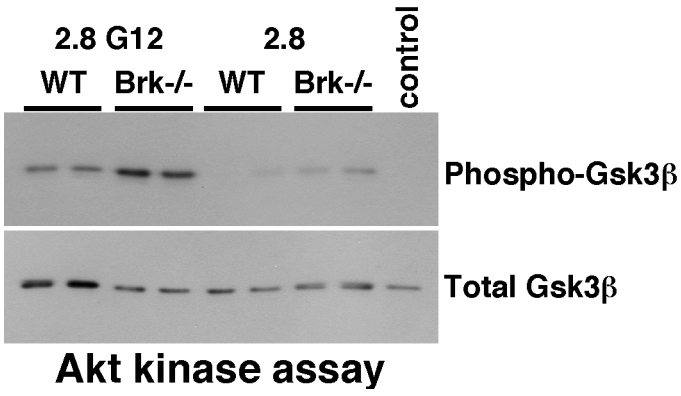

Fig. 10: Increased Akt kinase activity in the intestinal epithelium of Brk mutant mice. Endogenous Akt was immunoprecipitated from total cell lysates of the distal ileum of inbred (2.8 G12) and outbred (2.8) wild-type (WT) and knockout (KO) mice. *In vitro* kinase assays were performed with purified recombinant GSK-3 and ATP. Reactions were stopped at 0 (control) and 30 minutes and subjected to Western blotting with phospho-GSK-3β and total GSK-3β antibodies. Equal amounts of total GSK-3β protein were present in the reactions. Loss of Brk results in increased Akt kinase activity in the intestinal epithelium.

3.2. Brk signaling protects from intestinal inflammation

3.2.1. Chronic inflammation in Brk deficient mice

The regulation of proliferation of the stem cells in intestinal crypts is critical for the maintenance of the mucosal structure and the capacity for effective absorption and defensive barrier functions (Booth and Potten, 2001). Disruption of the balance between proliferation, differentiation and apoptosis leads to loss of normal absorptive functions and an increased risk of tumorigenesis.

Upon histological examination of Brk deficient mice, evidence for increased inflammation in multiple epithelial linings was observed. To investigate the intestinal immune homeostasis in Brk deficient mice, the entire length of the small and large intestine of Brk mutant mice was histologically examined by staining with hematoxylin and eosin, and monitored by light microscopy. Interestingly, an increased amount in lymphoid tissue was observed in knockout mice compared to wild-type controls (Fig. 11A). These organized mucosal lymphoid follicles, named Peyer's patches, were found at high frequency not only in the lower small intestine but also in the colon of Brk deficient mice (Fig. 11A-C). Peyer's patches are lymphoid follicles containing antibody producing B-cells to defend against invading bacteria, parasitic microbes, viruses and other foreign particles (Chen, et al., 2004). They normally appear in the mucus secreting lining of the small intestine as was observed in wild-type animals (data not shown). Peyer's patches not only play a role in the intestinal host defense but also in regulating immune homeostasis of the intestinal epithelium (Chen, et al., 2004). It has been reported that this modulation of epithelial physiology by Peyer's patch lymphocytes involves both cell-cell contact and cytokine signaling.

The observed increase in lymphoid tissue in Brk knockout mice suggested an increase in inflammation and activation of the immune system. Inflammation results

in production of pro-inflammatory cytokines and chemokines, and aberrant cytokine signaling is a hallmark of chronic inflammation (Philip, et al., 2004). Altered cytokine production appears to be critical for inducing pathologically increased rates of epithelial cell turn over in inflammation (Podolsky, 2002). Transcriptional upregulation of pro-inflammatory cytokines has been shown not only to be a result of a global response of intestinal epithelial cells to increased invasion with microbes and bacteria but can also be caused by imbalance in intestinal epithelial homeostasis (Pedron, et al., 2003).

To study cytokine production in Brk deficient mice, age-matched wild-type and knockout mice were sacrificed and the distal small intestine was used to prepare total RNA. RNase protection assays with a multi-probe for inflammatory cytokines were performed (Fig. 11B). Analysis of three wild-type and three knockout outbred (2.8) mice showed a significant increase in interleukin-18 (IL-18) and interleukin-6 (IL-6) mRNA levels in Brk knockout mice compared to wild-type controls (Fig. 11B). IL-6 has been shown to be a pro-inflammatory cytokine, which plays a dual role in protecting against mucosal injury and promoting intestinal tumorigenesis (Heinrich, et al., 2003). Increased IL-6 production has been shown to correlate with colon tumor formation and growth (Becker, et al., 2004). IL-18 is produced by various cells including intestinal epithelial cells. It has been shown to induce interferon-γ (IFN-γ), and to play a crucial role in proliferation and maintenance of the intestinal epithelium (Okazawa, et al., 2004). Increased levels of IL-18 have been found in intestinal mucosal biopsies from IBD patients (Monteleone, et al., 1999; Pizarro, et al., 1999). Thus, up-regulation of both IL-6 and IL-18 expression in Brk deficient mice supported the hypothesis of increased inflammation in knockout mice as indicated by the large amount of Peyer's patches.

Disruption of the mucosal barrier function and the concomitant immune hyperactivation by the microflora was shown to represent the key event that leads to the progressive transformation of epithelial cells and thus to chronic inflammation. To address this, the expression of proteins involved in maintaining barrier function was examined in the intestinal epithelium of wild-type and Brk deficient mice. To this extent, immunohistochemistry for the tight junction marker ZO-1 and the adherens type junction marker β-catenin were performed (Fig. 11C). Intestinal paraffin sections of wild-type and knockout mice were incubated with FITC-conjugated anti-ZO-1

antibodies and visualized by fluorescence microscopy. Comparing wild-type and Brk knockout mice for ZO-1 protein expression showed no significant difference in the appearance of tight junctions (Fig. 11C). Furthermore, immunohistochemistry for β-catenin was performed (Fig. 11C). Both wild-type and knockout animals showed a similar pattern and staining for membrane-bound β-catenin (Fig. 11C). Taken together, these data suggested no visible difference in epithelial barrier protein expression in Brk deficient mice and wild-type controls.

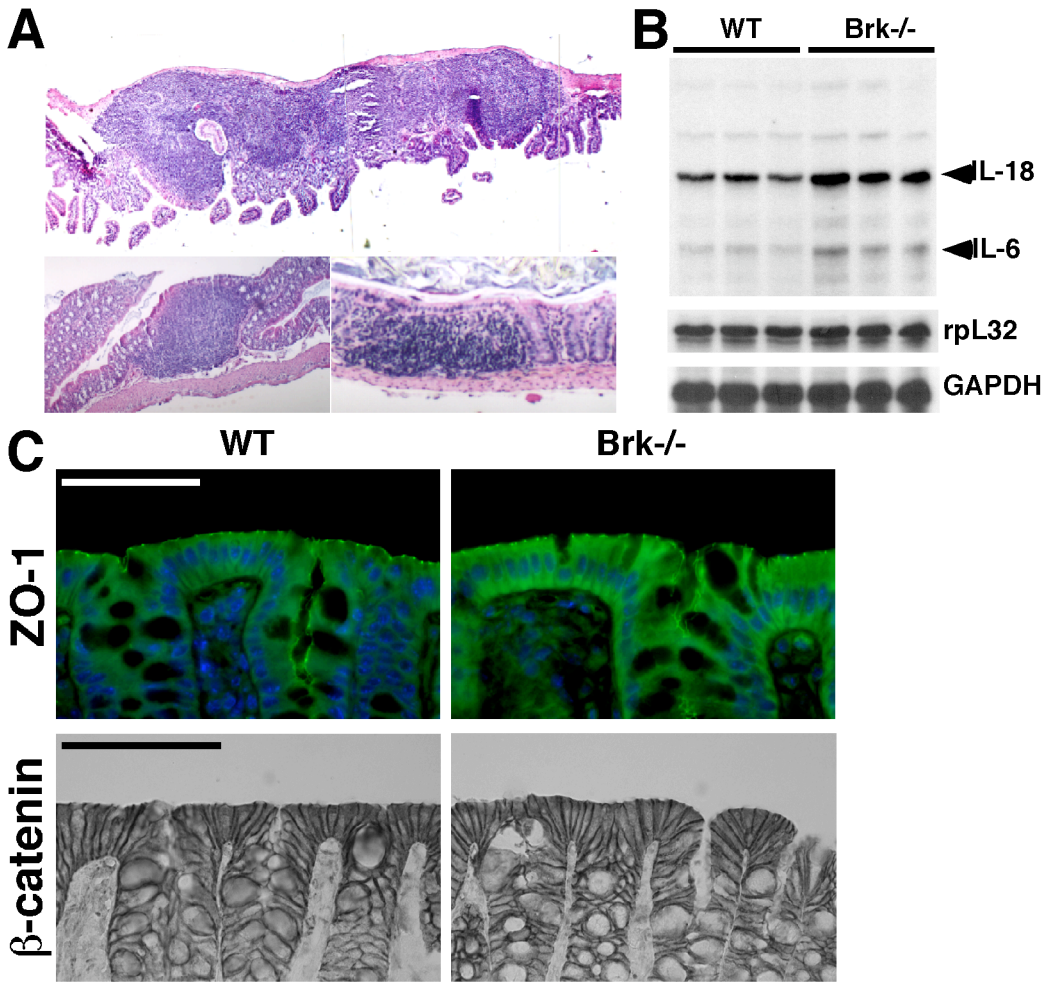

Fig. 11: Chronic inflammation in the intestinal epithelium of adult outbred Brk deficient mice (2.8). (A) Gross microscopical examination of intestinal sections from outbred 2.8 knockout mice. Sections from small intestine and colon were stained with hematoxylin/eosin. Increased amount of lymphoid tissue, Peyer's patches, was observed in mutant epithelium. (B) RNase protection assays were performed with total RNA from distal ileum of adult (8 weeks) wild-type (WT) and knockout (Brk-/-) oubred (2.8) animals and ^{32}P-labeled antisense multiprobes specific for cytokines. Rpl32 and GAPDH expression were examined as controls. Brk mutant mice exhibit increased expression of IL-18 and IL-6 in the intestinal epithelium. (C) Immunohistochemical analysis of the apical

epithelial barrier. Immunostaining with antibodies specific for ZO-1 and β-catenin on colonic sections from wild-type (WT) and mutant (Brk -/-) animals. No differences in the expression and position of these tight (ZO-1) and adherens (β-catenin) junction markers between wild-type and mutant mice were observed. Size bars represent 50 μm.

3.2.2. Brk protects the intestinal epithelium from cytokine-mediated injury

It has been reported that the balance of proliferation and differentiation along the crypt-villus axis is an important factor in protecting the intestinal epithelium from injury (Booth and Potten, 2001). Furthermore, it is well known that immune responses in the intestine remain in a state of controlled inflammation and that their deregulation leads to the development of inflammatory bowel diseases (Kanai and Watanabe, 2004).

To test the effect of loss of Brk on intestinal epithelial integrity, a model of intestinal injury and inflammation was chosen. This model utilized the oral administration of dextran sodium sulfate (DSS). DSS is a synthetic, sulfated polysaccharide that causes acute and chronic colitis (Seril, et al., 2003). The severity of induced injury greatly depends on the mouse strain analyzed, suggesting the influence of host genetic determinants on the inflammation severity and tumor risk. In general, genetically engineered mice on a C57BL background are more resistant compared with equivalent mutant mice on other genetic backgrounds such as 129/Sv.

Inbred Brk mutant and wild-type mice were subjected to 3% DSS in drinking water *ad libitum* for 5 days (5 days) followed by a recovery period with water for 3 days (8 days) (Fig. 12A). Both groups were closely monitored for weight loss and rectal bleeding, and at 5 or 8 days DSS treated mice were sacrificed and the gastrointestinal tract excised. The entire colon was fixed and embedded, and paraffin sections were stained with hematoxylin and eosin and evaluated by light microscopy. As seen from the comparison of representative photomicrographs taken of wild-type and Brk mutant mice at the indicated time-points, Brk mutant colons show severe and extensive denudation of the surface epithelium (erosions) and mucodepletion of glands compared to wild-type control mice (Fig. 12B,C). Separation of the crypt base from the muscularis mucosa and subsequent loss of crypts was observed in mutant mice at 5 days DSS, whereas a similar phenotype was not observed in wild-type mice

before 3 days post DSS. Furthermore, Brk mutant mice exhibited an extended amount of inflammatory infiltrate in their submucosa and lamina propria and a frequent appearance of focal erosions when compared to wild-type control mice (Fig. 12B,C). Especially at 3 days post DSS treatment an extensive inflammatory infiltration of the mucosa could be observed in Brk deficient mice (Fig. 12C). No difference in histological appearance was observed in untreated wild-type and Brk knockout colons (Fig. 12B).

In addition to the observed histological differences in wild-type and Brk deficient mice post DSS treatment, the expression of inflammatory cytokines was measured by performing RNase protection assays. Total RNA of untreated and DSS treated (8 days) colons of inbred wild-type and Brk knockout mice was prepared, and analyzed by multiprobe RNase protection assay. In contrast to our previous findings (Fig. 11B), no differences in cytokine mRNA levels were observed in untreated mice (Fig. 12D). This might be due to the genetic background of the analyzed animals. In contrast to the oubred mice (2.8) utilized for previous studies, inbred mice with a C57BL background (2.8G12) were used in the DSS study. Whereas genetically engineered mice on a 129/Sv strain appear to be especially susceptible to IBD-like disease, mice on a C57Bl background have been shown to be more resistant to inflammation (Rogers and Fox, 2004).

Significantly elevated levels of pro-inflammatory cytokines were observed post DSS treatment in both wild-type and knockout mice (Fig. 12D). However, an overall greater induction of cytokine mRNA was observed in Brk mutant mice when compared to wild-type control mice with DSS treatment (Fig. 12D). This upregulation of pro-inflammatory cytokine mRNAs in inbred Brk knockout mice suggested increased severity of acute inflammation in response to DSS treatment. Furthermore, loss of IL-18 expression, indicating the overall loss of epithelium, was observed in mutant mice.

Taken together, the mutant epithelium appeared more susceptible to DSS-induced histological damage than control epithelium accompanied by the enhanced production of many inflammatory mediators (Fig. 12A-D). This suggested that Brk signaling acts to ensure epithelial cell integrity and loss of Brk signaling results in increased epithelial susceptibility to injury.

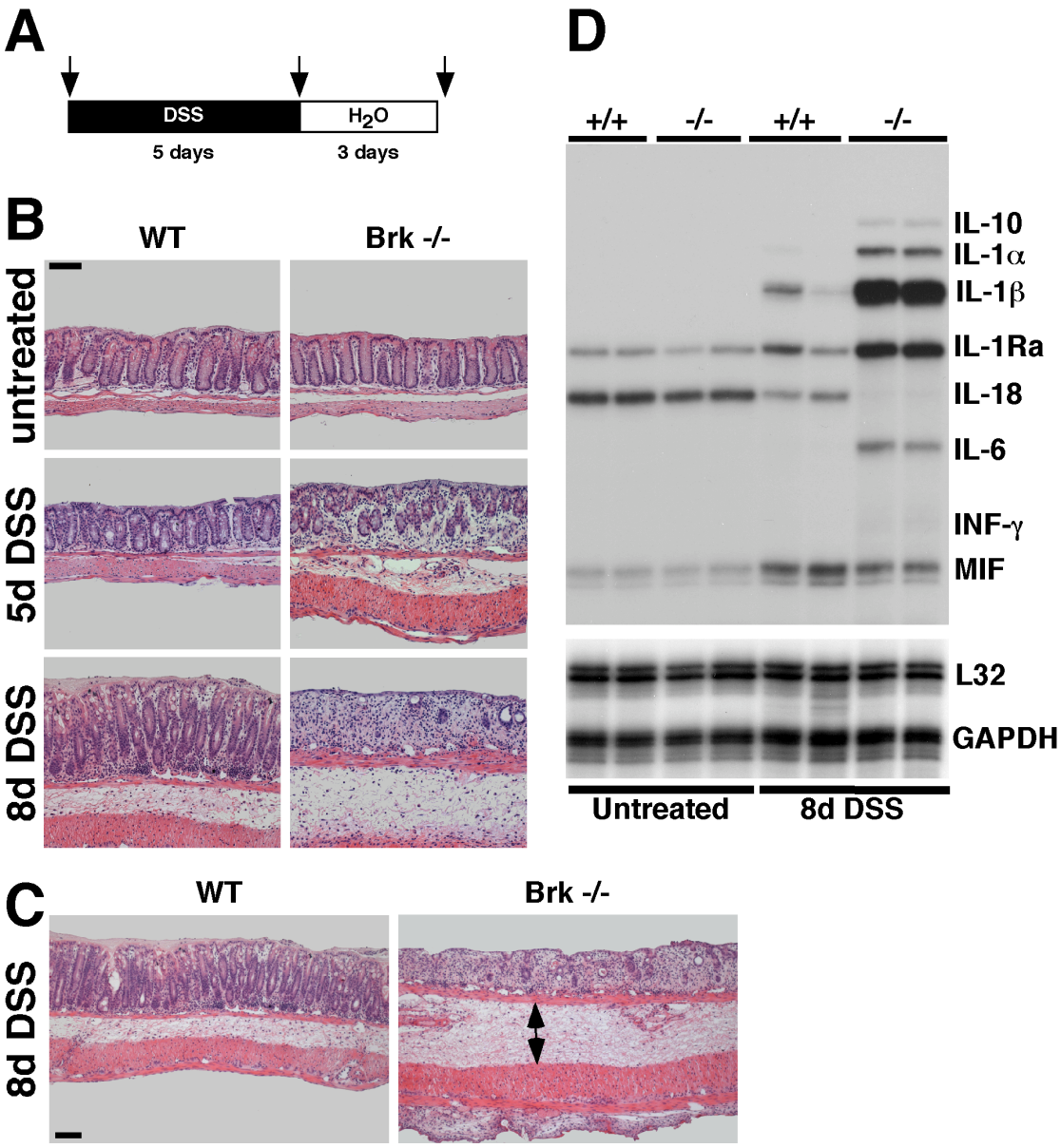

Fig. 12: Colonic epithelial damage in inbred Brk deficient mice following DSS administration. (A) Study design. Mice were subjected to 5 days of 3% DSS (5d) in drinking water followed by 3 days water (8d). Arrows indicate days 0, 5 and 8 of DSS treatment. (B, C) Representative photomicrographs of colons from inbred (2.8 G12) wild-type (WT) and knockout (Brk -/-) mice stained with hematoxylin/eosin at days 0, 5 and 8 of DSS treatment. Brk knockout mice exhibit increased epithelial injury with severe crypt loss after treatment. By day 8 after treatment, the inflammatory cell infiltration has become more extensive in mutant animals (indicated by arrow). Size bars represent 100 μm. (D) RNase protection assays were performed with total RNA from distal colon of inbred (2.8 G12) wild-type (WT) and knockout (Brk-/-) animals and ^{32}P-labeled antisense multiprobes specific for cytokines. Rpl32 and GAPDH expression was examined as controls. Brk mutant mice exhibit increased expression of pro-inflammatory cytokines, such as IL-6, but a decreased expression of the epithelial specific cytokines IL-18, in the colon after DSS treatment.

To elucidate the mechanisms behind the increased susceptibility of Brk deficient mice to DSS treatment, Brk protein expression in the intestine of wild-type mice during DSS induced colitis was analyzed. Ileum and colon mucosal lysates were prepared from the respective mice for Western blot analysis with anti-Brk-antibodies (Fig. 13A, B). Increased levels of Brk protein were observed in ileum and colon of wild-type mice treated with DSS compared to untreated mice (Fig. 13A, B). Consistent induction of Brk protein was observed in the ileum of treated wild-type mice at 5 days DSS and 3 days post DSS (8 days DSS) (Fig. 13A). However in colon, induction of Brk protein levels could only be detected in 5 days DSS mice whereas very low levels were present at 3 days post DSS treatment (Fig. 13B). This apparent loss of Brk protein is most likely due to the general loss of epithelial cells during the inflammatory process at the 8 day time-point. No signal was detected in Brk mutant mice, confirming their genotype.

To provide evidence that Brk is induced in epithelial cells post DSS treatment, Brk immunohistochemistry was performed. Colon sections of untreated, 5 days DSS and 3 days post DSS treated wild-type mice were immunostained with anti-Brk-antibodies and screened by light microscopy. Brk protein expression is restricted to non-proliferating cells in the top third of the intestinal epithelium in untreated wild-type mice (Fig. 13C). However, upon DSS treatment nuclear Brk is expressed throughout the crypt epithelium. Brk protein is excluded from inflammatory cells infiltrating the mucosa and the lamina propria (Fig. 13C, D). The observed strong nuclear localization of Brk suggested a possible role in the regulation of transcription in response to injury. Brk has been shown to phosphorylate and inhibit its nuclear substrate Sam68, which has been implied in the regulation of splicing (Derry, et al., 2000; Matter, et al., 2002). The physiological importance of this nuclear localization in response to injury needs to be further investigated.

In conclusion, Brk appeared to have a protective role in intestinal epithelial cells exposed to cytokines during inflammation. It may exert its protective function by promoting intestinal homeostasis, as loss of Brk expression resulted in increased proliferation. Thus, Brk signaling may be critical for the protection of gut injury and associated mortality.

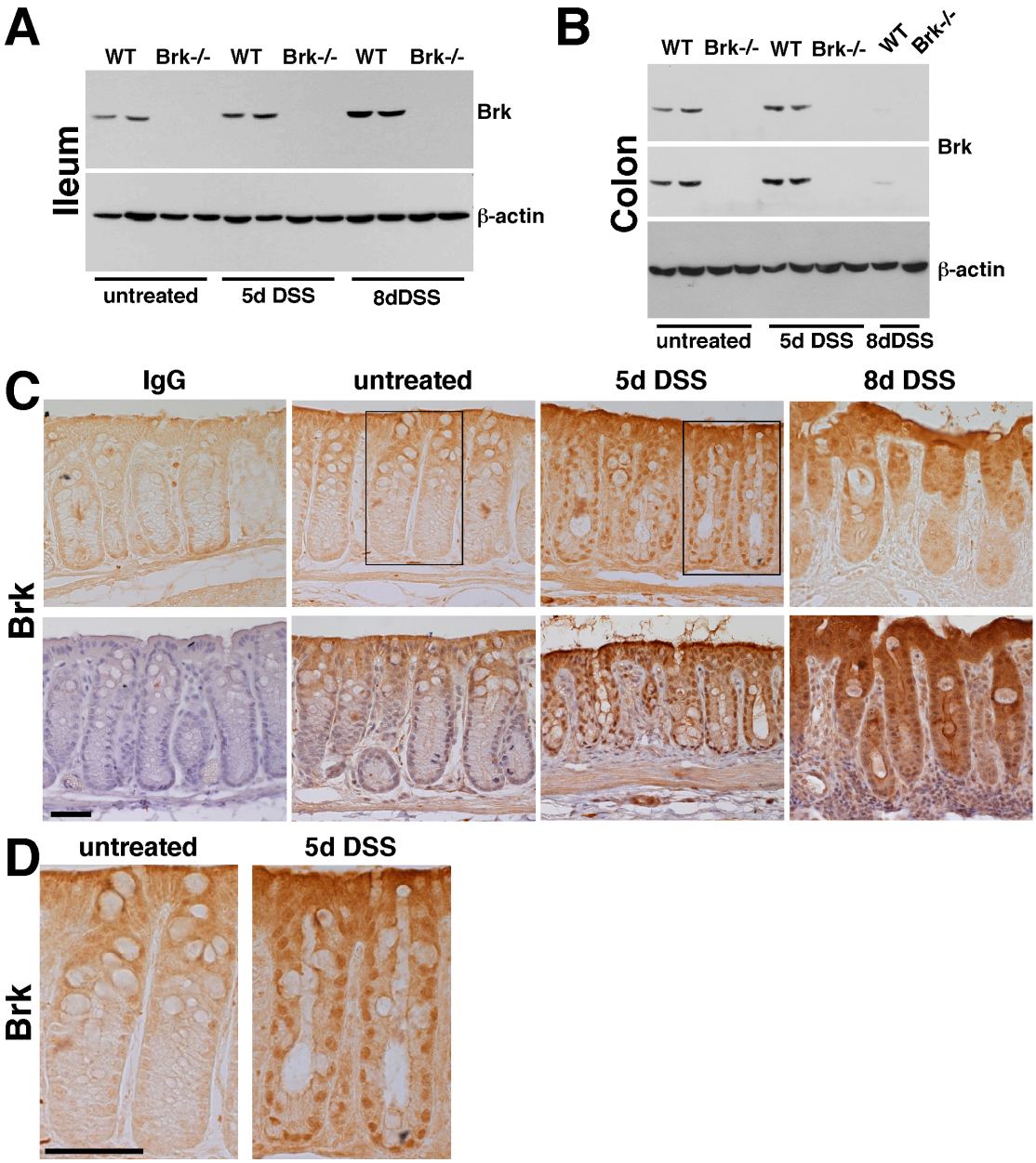

Fig. 13: Induction of nuclear Brk tyrosine kinase expression upon DSS treatment in wild-type mice. (A, B) Western blot analyses were performed with total cell lysate from distal ileum (A) and distal colon (B) of wild-type (WT) and knockout (Brk-/-) animals using antibodies against Brk. Expression of β-actin was examined as a control for protein loading. Brk protein expression increased with treatment. (C, D) Immunohistochemical analysis of colons from wild-type (WT) animals at days 0, 5 and 8 of DSS treatment with anti-Brk antibodies or anti-IgG antibodies as a control. Size bars represent 50 μm. Induction of nuclear Brk protein expression throughout the colonic epithelium of wild-type mice after DSS treatment.

3.2.3. Increased susceptibility of Brk deficient mice to DSS inhibits tumor development in the AOM/DSS tumorigenesis model

Increased proliferation, pro-survival signaling and chronic inflammation have all been correlated with the promotion of cancer (Philip, et al., 2004; Sancho, et al., 2004). The observation of these characteristics in Brk deficient mice led to the hypothesis that Brk may have tumor suppressor-functions in wild-type mice (Macleod, 2000; Sherr, 2004). Brk expression is restricted to non-proliferating cells in the intestinal epithelium. Loss of this tyrosine kinase in mice resulted in increased epithelial cell turnover and chronic inflammation, predisposing these mice to tumor development.

To get insights into Brk's putative tumor suppressor functions the AOM mouse model of colon carcinogenesis was utilized. AOM is a pro-carcinogen, which upon metabolic activation causes the formation of O^6-methylguanine (Pegg, 1984). It induces tumors in the distal colon of rodents and is commonly used to elicit colorectal cancer in experimental animals (Boivin, et al., 2003). In pilot studies, wild-type and knockout mice were subjected to weekly AOM injections (10 mg/kg) for the duration of 6 weeks and sacrificed 10 weeks after the last injection (Heap and Bie, unpublished). At that time, knockout mice exhibited a considerably higher incidence of aberrant crypt foci formation (ACFs) within the distal colon compared to wild-type controls. Aberrant crypt foci are the smallest identifiable lesions proposed to lead to colorectal carcinoma (Cheng and Lai, 2003). Furthermore, at 24 weeks after the last AOM injection, knockout mice exhibited colonic tumors, which were absent in wild-type mice (Heap and Bie, unpublished). These data supported a tumor suppressor function for Brk. However, due to the small number of animals used in these pilot experiments, further studies needed to be performed to confirm these preliminary results.

To this extent, wild-type and Brk deficient mice were subjected to a novel inflammation-related carcinogenesis model. In this accelerated tumorigenesis model, mice are subjected to a combination treatment of AOM and DSS, which represents an extremely efficient way to generate adenocarcinomas of the intestine, and greatly enhances tumor development. The inflammation caused by DSS treatment following AOM injection is presumed to represent the key event that creates a microenvironment, that is permissive for the progressive transformation of colon

epithelial cells, and greatly enhances the incidence of AOM-induced tumors (Okayasu, et al., 1996; Tanaka, et al., 2003). Mice subjected to this treatment develop tumors within 20 weeks of study begin. Age-matched wild-type and knockout mice were given a single intraperitoneal administration of AOM (10 mg/kg body weight), and a 1-week oral exposure to DSS (2%) (Fig. 14A). Twenty weeks post treatment mice were sacrificed and their entire colons were excised, opened longitudinally and fixed in 70% ethanol. Mice were closely monitored for weight loss during the entire study. Surprisingly, wild-type animals showed high mortality and morbidity upon treatment with a loss of 50% of treated mice (Fig. 14B). In contrast, Brk knockout mice showed 80% survival and lower morbidity upon treatment compared to wild-type controls (Fig. 14B). Furthermore, after week 12 of the study, an increased amount of wild-type mice with anal prolapse due to tumor development in the distal colon were observed.

To further analyze these surprising results, the colons of wild-type and knockout mice that survived untill the end of the study were macroscopically examined by light microscopy. Nodular and polyploid colonic tumors were observed in the middle and distal colon of wild-type mice (Fig. 14C,D). In sharp contrast, the number and size of tumors were substantially reduced in Brk mutant mice when compared to wild-type mice (Fig. 14C,D). Thus, despite the appearance of chronic inflammation in Brk deficient mice and in contrast to our pilot study, the colitis-associated tumor incidence in the DSS/AOM model was greatly reduced in Brk deficient mice. However, it was previously observed that the Brk mutant epithelium was more susceptible to DSS-induced histological damage than control epithelium (Fig. 13). Brk signaling appeared to be critical for the protection against DSS-induced inflammation, and treatment of Brk knockout mice with DSS resulted in an immediate and major upregulation of pro-inflammatory cytokines including IL-6. This is considered a hallmark of acute inflammation, which has been shown to counteract cancer development. This observation suggested that treatment of knockout mice with 2% DSS after the initial AOM injection resulted in acute inflammation in the colonic crypt and henceforth in major loss of crypt epithelium in Brk deficient mice. This loss of crypt epithelial cells, which were transformed by AOM, may be the cause for the lower tumor incidence in Brk deficient mice.

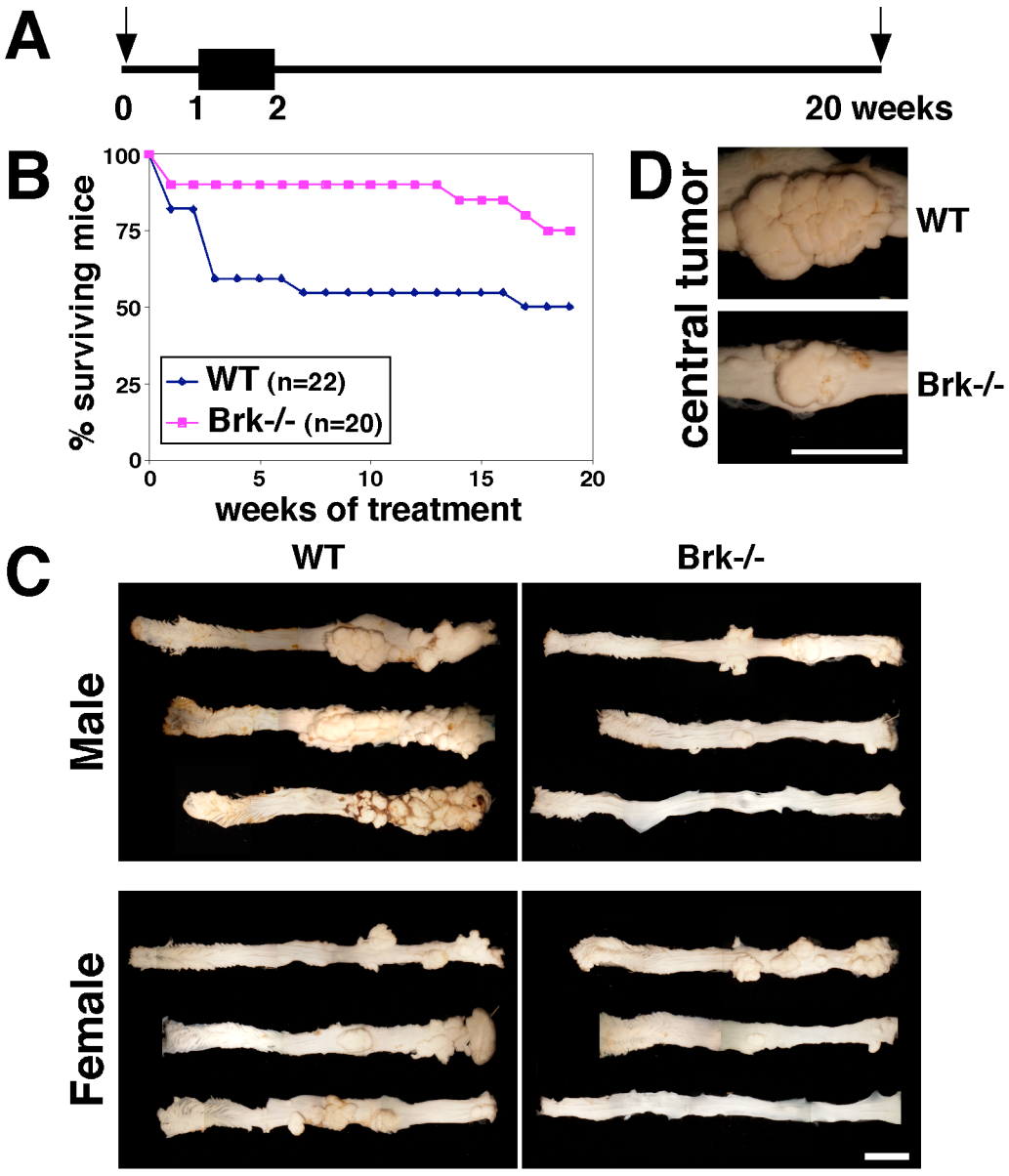

Fig. 14: Inflammation-related mouse colon carcinogenesis model. (A) Study design. Arrows indicate initial AOM injection at 0 weeks and study end at week 20 respectively. The black box indicates the 7 day treatment with 2% DSS. (B) Increased mortality in wild-type mice upon treatment. Survival of wild-type (WT) and mutant (Brk -/-) mice was monitored over time of treatment. (C, D) Macroscopic view of colons of wild-type (WT) and mutant (Brk -/-) mice after AOM/DSS treatment. Size bars represent 1 cm. Due to their increased susceptibility to inflammation, Brk deficient mice appear less susceptible to AOM/DSS induced tumor formation in the colon.

In wild-type mice on the other hand, tumor formation is promoted by the induction of inflammation by 2% DSS after the initial low dose of AOM, henceforth resulting in the high tumor incidence reported. To prove this hypothesis and support a tumor suppressor function for Brk and hence validate the previous pilot study, further

experiments are needed. Studies with a lower dose of DSS (0.5%) combined with AOM and AOM only carcinogenesis models are underway.

3.3. Brk sensitizes cells to apoptosis *in vivo* and *in vitro*

3.3.1. Brk is required for DNA-damage induced intestinal apoptosis

Apoptosis is a cellular response that regulates important processes such as tissue homoeostasis, defense against certain pathogens and elimination of unwanted cells. The imbalance in proliferation and differentiation in the gastrointestinal tract of Brk deficient mice suggested that Brk is involved in the maintenance of tissue homeostasis. Brk knockout mice exhibited an expanded progenitor zone and the physiological appearance of longer villi. Since apoptosis is a mechanism involved in counterbalancing the crypt cell division (Potten, 1992), one could hypothesize that Brk deficient mice may be impaired in their apoptotic response. The small intestinal epithelium provides a well-characterized, readily accessible and quantifiable model to address questions regarding the importance of certain gene products on epithelial cell fate *in vivo* following genotoxic damage (Clarke, et al., 1994; Merritt, et al., 1997; Merritt, et al., 1995; Merritt, et al., 1994; Potten, 1992). Following genotoxic damage, cell death is induced in the crypt compartment (Potten, 1990).

To investigate a potential role of the Brk tyrosine kinase as a physiological regulator of apoptosis in mouse intestinal epithelia, Brk deficient mice were analyzed in their spontaneous or damage-induced apoptotic response. Wild-type and Brk deficient mice were either untreated or subjected to whole-body γ-irradiation (8 Gγ), which has been shown to induce apoptosis in epithelial cells of the small intestinal crypts (Potten, 1990). Apoptotic cells were identified by caspase 3 activation by immunostaining with anti-cleaved caspase 3-antibodies, and quantified microscopically on a cell positional basis. Previously, an increase in epithelial cell proliferation marked by increased BrdU labeling in the intestine of Brk deficient mice was noted (Fig. 6). This extended zone of proliferation correlated with the histological appearance of longer villi, suggesting that the increased proliferation in Brk deficient mice is not counterbalanced by an increased cell loss. In keeping with this observed intestinal phenotype of Brk knockout mice, no statistically significant differences in spontaneous apoptosis within the crypt epithelia of untreated wild-type and Brk

knockout mice were observed (data not shown). Apoptotic small intestinal crypt cells were infrequent in both non-irradiated wild-type and Brk null mice.

However, treatment with γ-irradiation induced profound apoptosis in proliferating areas of small intestinal crypts in wild-type but not Brk deficient mice observed at 6 and 72 hours after γ-irradiation (Fig. 15A). With the dose of 8 Gγ, the 6 hour time-point represents the peak of early apoptosis in response to γ-irradiation in the small intestine (Potten, 1997, Potten, 1998 #3408). The 72 hour time-point on the other hand represents the time of crypt regeneration and repopulation, when apoptotic crypt cells are migrating towards the apex of the villus where they will be shed. Brk deficient mice exhibited significantly less apoptosis than their wild-type counterparts with a 2-fold decrease in number of apoptotic cells compared to wild-type mice at both time-points (Fig. 15B). Wild-type mice on the other hand exhibited 4 to 6 apoptotic events per crypt/villus unit consistent with the saturation level of small intestinal apoptosis reported in the literature (Fig. 15B) (Potten, 2004). By Student's t-test the observed differences were highly significant ($P \leq 0.05$). In addition, loss of Brk resulted in a reduced percentage of crypt-villus units that showed significant epithelial apoptosis (4 or more apoptotic cells per unit) (Fig. 15C). The observed reduction of apoptotic cells in Brk deficient mice at both 6 and 72 hours post irradiation suggested that the differences at the early time-point are not due to a delay in the apoptotic response in knockout mice. To support these findings, tissue lysates from distal ileum of wild-type and Brk deficient mice at 6 hours post irradiation were prepared for Western blotting. Less cleavage of caspase 3 detected by Western Blotting was observed in Brk deficient mice compared to wild-type mice (Figure 15D).

These data suggested that the non-receptor tyrosine kinase Brk is involved in the intestinal DNA-damage induced p53-dependent apoptosis *in vivo*. Therefore, Brk might be an important determinant of damage-induced apoptosis in intestinal epithelia.

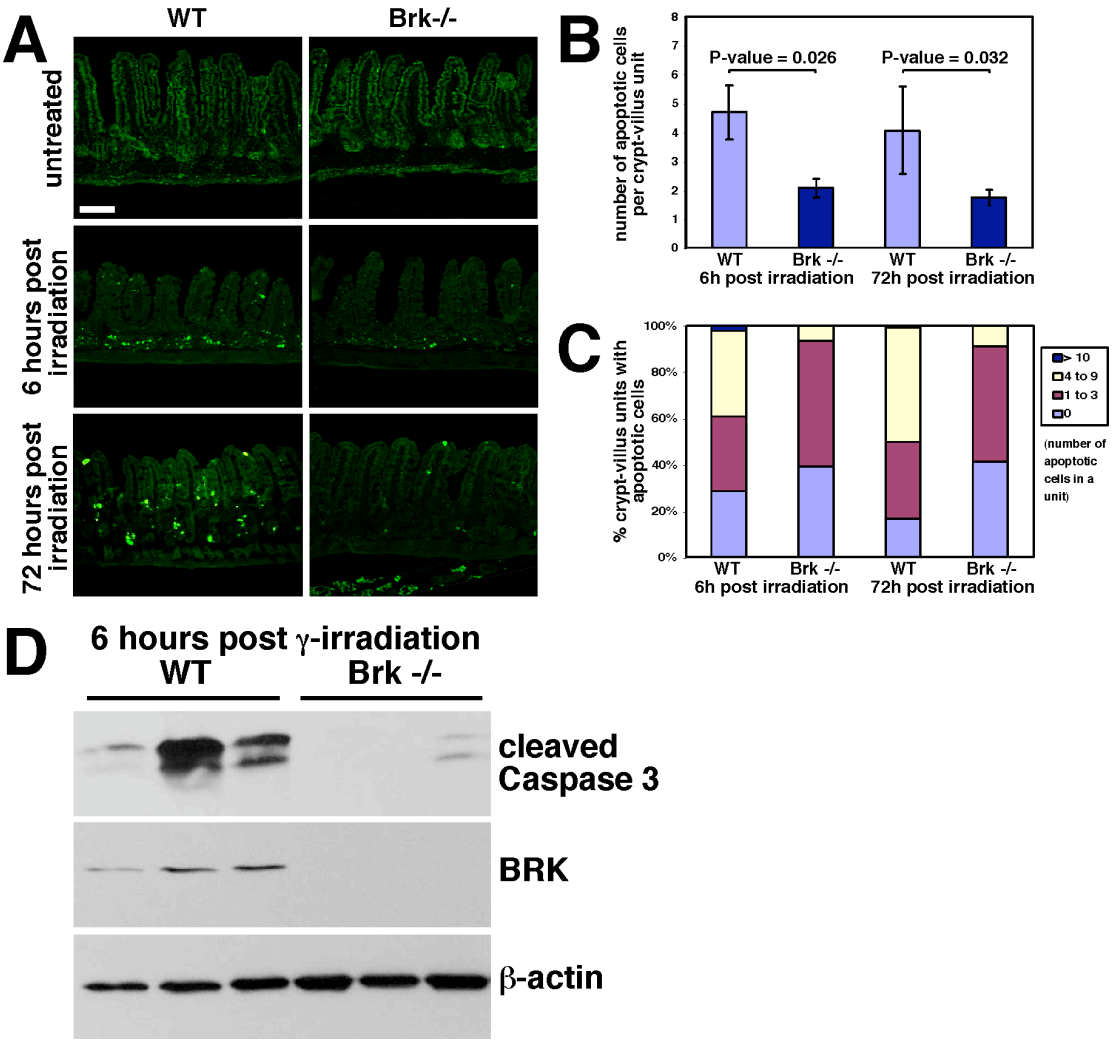

Fig. 15: Resistance to DNA-damage induced apoptosis in the absence of Brk. (A) Radiation –induced apoptosis of cells in the small intestine. Sections of distal jejunum from untreated and γ-irradiated (8 Gγ) wild-type (WT) and knockout (Brk -/-) mice at 6 and 72 hours post irradiation were prepared, analyzed by staining for cleaved caspase 3 and detected with Avidin-FITC (apoptotic cells stain green). Size bar represents 100 μm. (B, C) Histogram (B) and frequency histogram (C) of apoptotic cells per crypt-villus unit. Approximately 100 crypt-villus units per section were scored. Values shown are mean ± S.D. from two sections from at least three different mice per group. (D) Western blot analysis of irradiated ileum. Total cell lysates from distal ileum of wild-type (WT) and knockout (Brk -/-) mice 6 hours after irradiation (8 Gγ) were subjected to Western blotting with anti-cleaved caspase 3 antibodies. Immunoblotting with anti-Brk and anti-β-actin antibodies served as controls for the genotypes and protein loading respectively. Brk deficient mice show increased resistance to radiation-induced apoptosis in the small intestine.

3.3.2. Enhanced pro-survival Akt and MAPK signaling in Brk knockout mice

As previously observed, untreated mice deficient for Brk exhibited enhanced Akt/ Protein Kinase B activation when compared with wild-type control mice (Fig. 10). The Akt serine/threonine kinase has been shown to act as a survival factor that stimulates progression of the cell cycle and prevents cells from undergoing apoptosis (Datta, et al., 1997; Weng, et al., 2001; Weng, et al., 2001)}. To investigate whether the reported enhanced Akt activation in knockout mice might contribute to the apoptotic resistance of these mice to irradiation, lysates from distal ileum of wild-type and Brk mutant mice were analyzed by Western Blotting and Akt *in vitro* kinase assays.

Tissue homogenates of age-matched wild-type and knockout mice at 6 hours post irradiation were separated by SDS-PAGE, and Western Blotting with anti-phospho-Ser473-Akt and anti-total Akt antibodies was performed (Fig. 16). Phosphorylation of Akt at both serine 473 and threonine 308 is required for complete activation of this kinase. During activation, Akt undergoes a conformational change exposing these sites to phosphorylation. However, phosphorylation of Thr308 is more transient than Ser473. Total tissue lysates of wild-type and Brk deficient mice contained similar amounts of total Akt protein whereas increased amounts of Akt activation by Ser473 phosphorylation were detected in mutant ileum compared to wild-type controls (Fig. 16A). To confirm these data, total Akt was immunoprecipitated from tissue lysates, and the phosphorylation state was determined by anti-phospho-Ser473 antibodies. Total Akt recovered from Brk mutant ileum showed increased Ser473 phosphorylation compared to wild-type animals (Fig. 16A). Ser-phosphorylation of Akt was detected in wild-type mice but it occurred at much lower levels compared to the knockout counterparts (Fig. 16A). Similarly, using a two-step *in vitro* kinase assay to determine Akt activity, only Akt precipitated from tissue homogenates of Brk deficient mice resulted in major phosphorylation of GSK-3, whereas GSK-3 phosphorylation levels in wild-type animals were markedly reduced (Fig. 16B).

Exposure of cells to ionizing radiation and a variety of other toxic stresses has been reported to induce simultaneous compensatory activation of MAPK signaling pathways. In addition to the radioprotective and growth-promoting signaling executed

by the PI3-K/Akt pathway, ERK pathway signaling has been shown to be induced by radiation and to further stimulate an anti-apoptotic response (Dent, et al., 2003). To delineate the signaling pathways involved in the observed pro-survival response in Brk deficient mice, total tissue lysates were further analyzed by Western blotting. Lysates from distal ileum of untreated and irradiated (6 and 72 hour time-points) wild-type and Brk knockout mice were collected for Western blot analysis with anti-phospho-Erk1/2, anti-total-Erk1/2, anti-Brk and anti-β actin antibodies. Irradiation greatly stimulated activation of Erk1/2 MAPK in Brk deficient mice but not in wild-type counterparts at both time-points studied post treatment (Fig. 16C). Taken together with the previously described increased activation of Akt signaling in these mice, disruption of Brk expression stimulated anti-apoptotic Akt/PKB and Erk1/2 signaling in the gastrointestinal tract after γ-irradiation, contributing to and/or resulting in increased resistance to radiation-induced apoptosis.

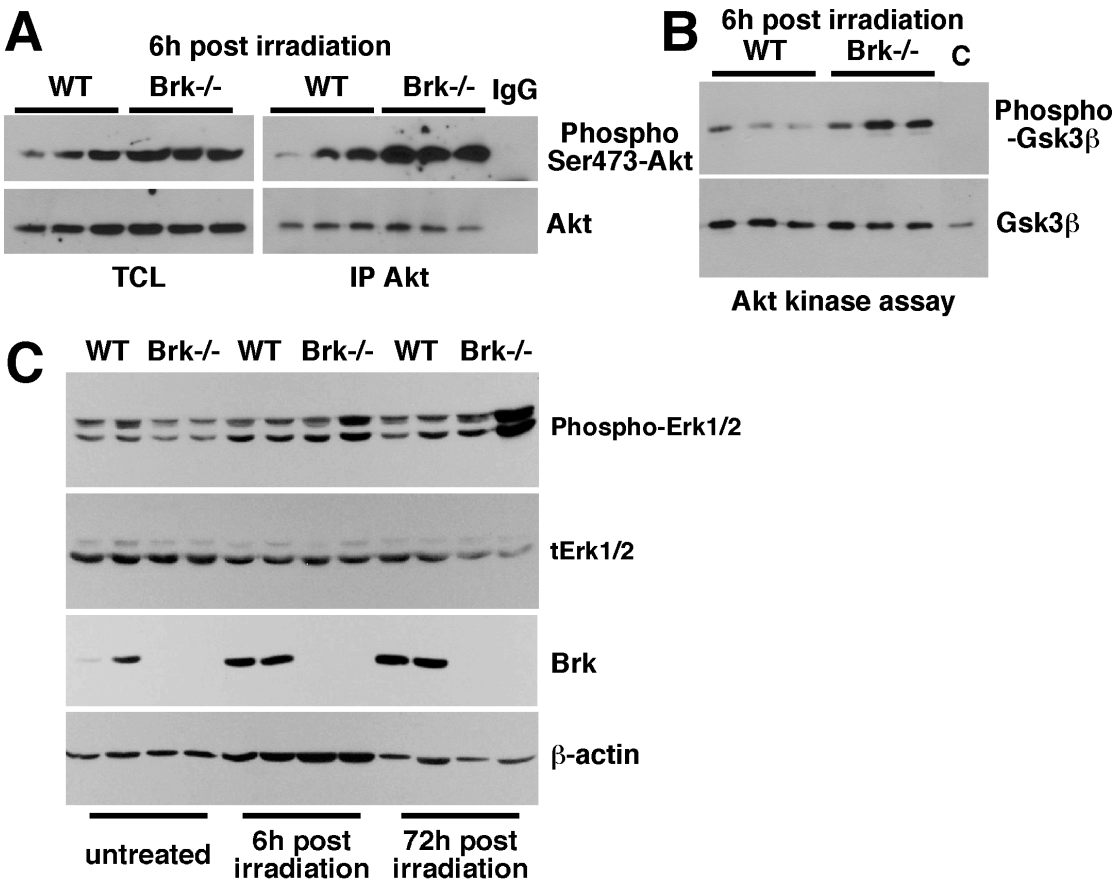

Fig. 16: Pro-survival signaling in Brk deficient mice. Total cell lysates (TCL) from distal ileum of wild-type (WT) and knockout (Brk -/-) animals at 0, 6 and 72 hours after whole-body γ-irradiation (8 Gγ) were prepared. (A) Lysates from

irradiated (6 hours) wild-type and knockout mice were analyzed by Western blotting and immunoprecipitation (IP). Endogenous Akt was immunoprecipitated and subjected to Western blotting with phospho-Akt specific antibodies. IP for IgG served as a control. Equal amounts of Akt were present in each immunoprecipitation as confirmed by immublotting for total Akt. Brk deficient mice exhibit increased phospho-Ser473 Akt. (B) Akt *in vitro* kinase assays. The lysates analyzed in (A) were subjected to Akt kinase assay. Endogenous Akt was immunoprecipitated with immobilized Akt antibody, and incubated with purified recombinant GSK-3 and ATP in kinase reaction buffer. Reactions were stopped at 0 (control or C) and 30 minutes and subjected to immunoblotting with Phospho-GSK-3β and total GSK-3β antibodies. Increased Akt kinase activity is detected in mice deficient for Brk. (C) Western blot analysis of TCL from untreated and irradiated (6 and 72 hours) wild-type and knockout mice with antibodies against phospho- and total Erk1/2. Expression of Brk and β-actin were examined as controls for genotype and protein loading respectively. Increased phosphorylation of Erk1/2 was observed in Brk deficient mice.

3.3.3. Induction of Brk in the intestine following ionizing radiation

It was previously reported that Brk protein levels are induced after DSS treatment. To determine whether irradiation induces Brk expression in intestinal epithelial cells *in vivo*, wild-type mice were exposed to whole-body γ-irradiation. The gastrointestinal tract was excised, total RNA and protein were prepared from distal ileum and analyzed by RNase protection assays and Western blotting. Tissue was also processed for immunohistochemistry.

Analysis of Brk mRNA levels using an anti-sense probe for Brk revealed no differences in Brk steady-state mRNA levels upon irradiation in wild-type animals (Fig. 17A). However, Brk protein levels rapidly increased after γ-irradiation in a time dependent manner detected by Western blotting with anti-Brk antibodies (Fig. 17B). To confirm these data, Brk protein expression in the small intestine of untreated and irradiated wild-type mice was furthermore examined by immunohistochemistry. Paraffin sections of the distal jejunum were immunostained with anti-Brk antibodies and counterstained with hematoxylin. As controls, sections were incubated with anti-rabbit IgG instead of anti-Brk antibodies. Untreated mice showed the previously described restriction of Brk protein expression to non-proliferating, terminally differentiated cells of the villus (Fig. 17C). In contrast, Brk protein expression was detected not only in the villus but also in proliferating cells of the crypt compartment in wild-type mice treated with ionizing radiation (Fig. 17C). The additional

expression of Brk protein in cells, which usually exclude its presence, could account for the increased protein levels detected by Western blotting in irradiated wild-type mice. The rapid radiation-induced upregulation of Brk protein levels but not mRNA indicated that Brk regulation at the protein level is a determinant of cellular sensitivity to genotoxic stress.

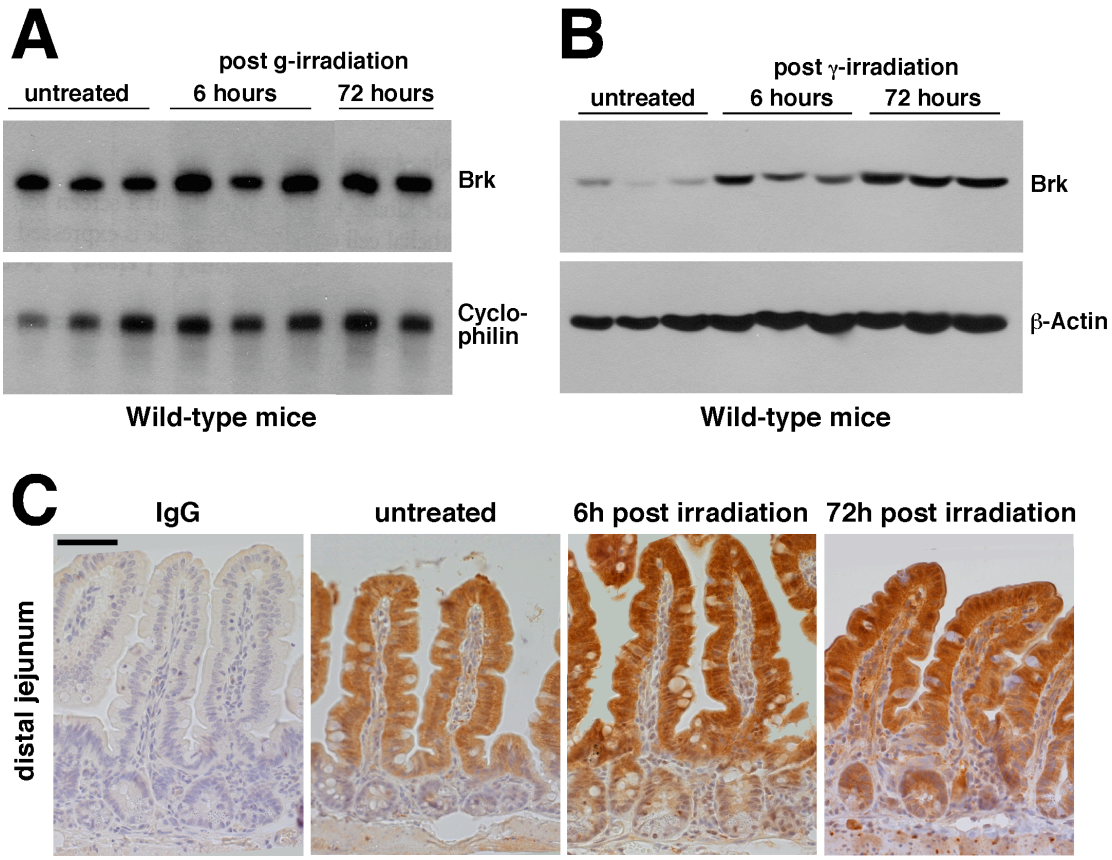

Fig. 17: Brk protein expression is induced by γ-irradiation. (A) RNase protection assays were performed with total RNA from distal ileum of untreated and irradiated wild-type animals and ^{32}P-labeled antisense probes specific for Brk and cyclophilin as control. (B) Western blot analysis of untreated and irradiated wild-type mice with lysates from distal ileum and antibodies against Brk and β-actin as a loading control. Increased Brk protein levels were detected in irradiated mice. (C) Immunohistochemical analysis of Brk expression on distal jejunum sections of untreated and irradiated wild-type animals. Immunostaining for IgG served as a control. Brk protein expression is restricted to the villus of untreated mice, whereas it is also detected in the crypt of irradiated mice. Size bar represents 50 *μ*m.

3.3.4. Expression of Brk sensitizes cells to apoptosis *in vitro*

To understand the role of Brk in the regulation of differentiation and apoptosis, Rat1a fibroblasts stably overexpressing Brk were generated. Rat1a cells are immortal nontransformed rat embryo fibroblasts that are susceptible to oncogenic transformation and exhibit serum- and Akt-dependent susceptibility to a variety of apoptotic stimuli (Topp, 1981). They do not express endogenous Brk, but have been shown to take up DNA efficiently and to this extent, Rat1A cells were transfected with either retrovirus containing a BRK expression plasmid encoding for the wild-type or activated Brk tyrosine kinase (Brk WT or Brk Y-F) or the corresponding empty vector (pLXSN), followed by selection in G418. As expected, the correct Brk protein was expressed in the former but not in the latter stable populations (Fig. 18A). Ectopic Brk expression appeared to have no effect on mean cell size.

The growth properties of the established polyclonal Rat1A cell lines were examined by growth rate and FACS analysis. The cell cycle profiles of Rat1A fibroblasts overexpressing Brk were analyzed by flow cytometry and BrdU incorporation (Fig. 18B). Cells were synchronized in 0.1% FBS for 48 hours resulting in cell cycle arrest in the G1 phase of the cell cycle (Fig. 18B). Following cell cycle arrest, cells were released into normal growth medium containing 10% FBS and the amount of cells entering S-phase was monitored at various time points post stimulation. No difference was observed in the release into the cell cycle and the amount of cells entering S-phase in fibroblasts ectopically expressing wild-type or activated Brk and the control cell line (Fig. 18B). Interestingly, following cell cycle arrest by growth in 0.1% FBS for 48 hours, cell lines expressing wild-type or activated Brk exhibited more cells in the sub G1 phase of the cell cycle than the control line expressing vector only (Fig. 18B). The sub G1 phase represents apoptotic cells with nuclear fragmentation.

For growth rate determinations, cells of the respective stable populations were seeded at the same density, and cell numbers were determined daily for 5 days. No profound differences in growth were observed between fibroblasts overexpressing wild-type and activated Brk or the empty vector only (Fig. 18C). It appeared that their doubling time was similar. However, control cells grew to higher saturation density than wild-type and activated Brk expressing Rat1a cells, as was apparent with the growth curve at 5 days of growth (Fig. 18C). Rat1A cells undergo contact inhibition

and G1 arrest when confluent. The decrease in cell number in Brk expressing populations once they reach confluence could be attributed to either a difference in cell morphology or increase in cell death compared to control populations. Brk has been shown to phosphorylate paxillin, and through this pathway it promotes cell motility and migration (Chen, et al., 2004). However, no clear difference in cell morphology was observed.

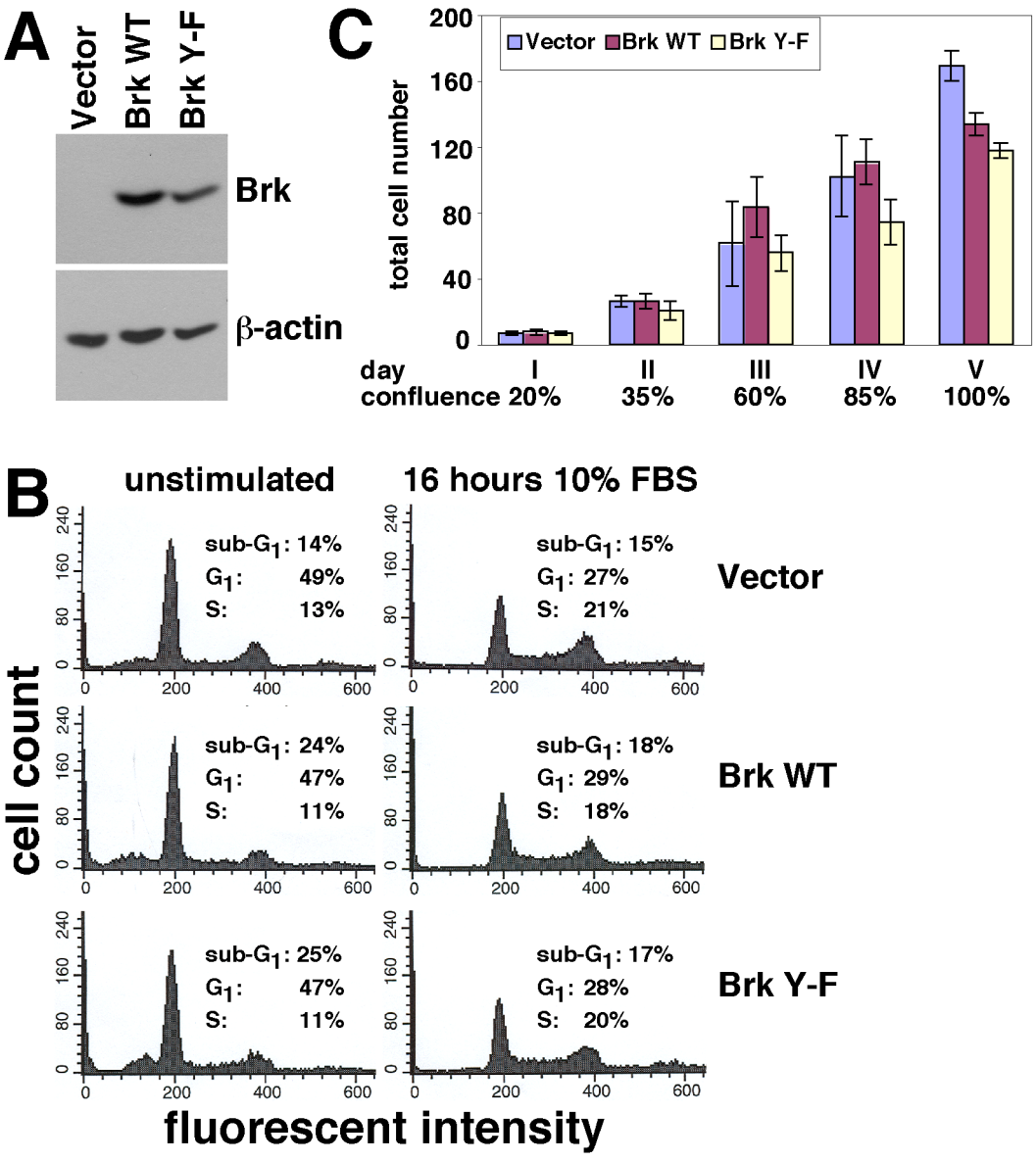

Fig. 18: Growth properties of stable Rat1A cells. (A) Western blot analysis of stable Rat1A fibroblasts. Total cell lysates of Rat1A cells stably overexpressing the expression vector pLXSN (Vector), wild-type Brk (Brk WT) and constitutively activated Brk (Brk Y-F) were analyzed by immunoblotting with anti-Brk antibodies and anti-β-actin antibodies as a control for protein loading. Rat1A cells do not express endogenous Brk. (B) Cell cycle analysis. Stable Rat1A

populations were synchronized and arrested in G_0 by serum starvation for 48 hours. Cells were released into G_1 with the addition of 10% FBS, harvested at 0 and 16 hours post stimulation, and analyzed for cellular DNA content by flow cytometry. (C) Exponential growth curves of the indicated stable Rat1A populations. The total amount of cells per 6 cm plate was counted for 5 consecutive days. Values are means ± S.D. of 2 experiments with at least 3 plates per cell line and time point. No differences in growth and cell cycle were observed in Rat1A fibroblasts stably overexpressing Brk WT or Brk Y-F.

To investigate a possible role of Brk in apoptosis and cell survival *in vitro*, the effect of serum deprivation on stable Rat1A fibroblasts expressing wild-type and activated Brk or the expression vector alone was examined (Fig. 19A-D). Respective cells were seeded at the same density, serum starved in 0% FBS for 24 hours and fixed followed by DAPI staining. The amount of cells with condensed nuclei, typical of apoptotic cells, was counted and the data plotted (Fig. 19B). Clearly, overexpression of wild-type and a constitutively active form of Brk resulted in serum starvation-induced apoptosis. Serum deprivation resulted in rapid cell death of cell lines with ectopic Brk, with 15-20% cell death within 24 hours (Fig. 19B). In contrast, control cells stably expressing the empty expression vector exhibited nuclear condensation similar to untreated populations when exposed to serum deprivation (Fig. 19B). A background level of spontaneous apoptosis of approximately 5% was observed in all stable populations.

These data were confirmed by FACS analysis of untreated and serum starved Rat1A populations (Fig. 19C). The cell lines were treated as described above and the amount of apoptotic cells was determined by staining with propidium iodide and subjecting the cells to flow cytometry. Apoptotic cells are represented by cells in the sub G1 phase of the cell cycle due to nuclear fragmentation. All untreated cell populations exhibited a similar and minimal amount of cells in sub G1 consistent with the low background level of apoptosis observed earlier. However, after serum starvation, cells overexpressing Brk exhibited a significantly greater amount of cells in sub G1 compared to control cells (Fig. 19C). Consistent with the data acquired by DAPI staining, Rat1A fibroblasts with ectopic Brk seemed to have 3 times more apoptotic cells compared to the control cell population (Fig. 19B, C). Taken together these data suggested that Brk sensitizes Rat1A fibroblasts to serum starvation induced apoptosis.

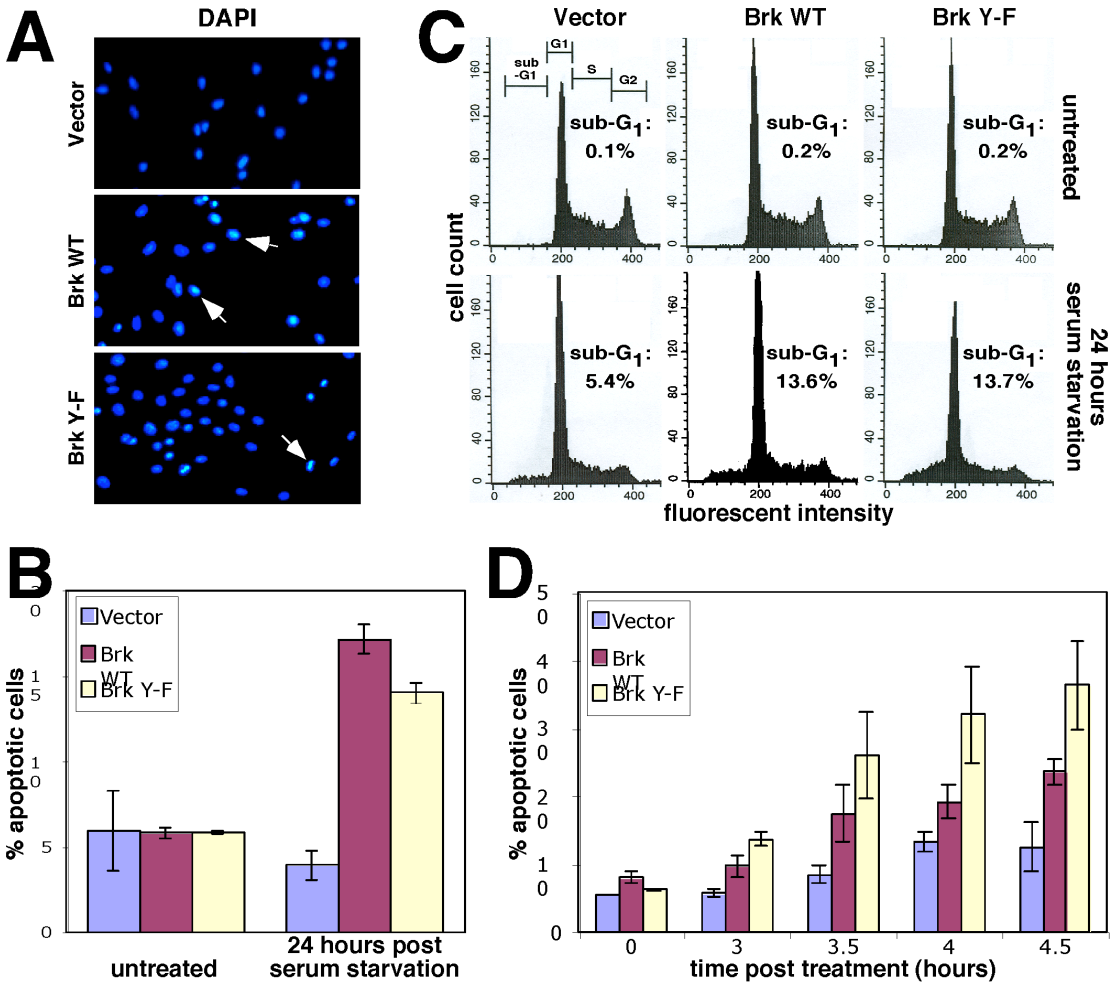

Fig. 19: Brk sensitizes Rat1A fibroblasts to apoptosis induced by serum starvation or UV-irradiation/serum starvation. Stable Rat1A populations overexpressing vector alone (Vector), wild-type (Brk WT) and activated Brk (Brk Y-F) were plated at equal density and apoptosis was induced by 24 hours serum starvation or by serum starvation and ultraviolet irradiation (50 J/m^2). Control cells were grown in 10% FBS. The percentage of cells with condensed chromatin was determined by DAPI staining. (A) Representative photomicrographs of DAPI-stained populations after 24 hours serum starvation. The arrows indicate cells with condensed chromatin (B) Histograms of percentage of apoptotic cells in untreated and serum starved populations. Percentage of apoptotic cells was determined microscopically after staining cells with DAPI. The values are means ± S.D. of three independent experiments. (C) FACS analysis of apoptosis. Untreated and serum starved populations were fixed, stained with propidium iodide (PI) and subjected to FACS analysis. The percentages of cells in sub G1 are indicated. (D) Cells were subjected to UV irradiation and at the time points indicated, chromatin condensation and the percentage of apoptotic cells were determined by DAPI staining. Values represent the average of three independent experiments ± S.D. Cell lines expressing Brk show an earlier onset of apoptosis and undergo apoptosis to a greater extent when compared to control cell lines (vector).

Another method to induce apoptosis in Rat1A fibroblasts in a controlled fashion is a combination of serum deprivation with a low dose of UV irradiation (Kennedy, et al., 1999). As has been previously shown, this combination accelerates growth factor-withdrawal-induced apoptosis and provides a convenient time frame for the analysis of the temporal sequence of apoptotic events. Serum deprivation alone induces apoptosis in Rat1A fibroblasts only after 24-36 hours. Furthermore, the dose of ultraviolet (UV) irradiation that is being used does not induce apoptosis in the presence of serum, whereas the combination of the two induces apoptosis after 5 hours (Kennedy, et al., 1999). To further characterize the sequential events leading to increased apoptosis in serum starved Brk expressing Rat1A cells, stable Rat1a fibroblasts were subjected to serum deprivation and UV exposure and analyzed. Following treatment, cells were fixed at the indicated time points, and the amount of apoptotic cells was determined by DAPI staining (Fig. 19D). The results showed a background level similar to the previous experiment with approximately 5% apoptosis in all cell lines. However, an increased amount of apoptotic cells was observed already at 3 hours post treatment in wild-type and Brk Y-F expressing fibroblasts. In contrast, the number of apoptotic cells did not increase until later, at around 4 hours post treatment in the control cell line (Fig. 19D). This earlier onset of cell death in populations with ectopic Brk was coupled with an increased amount of cell death compared to control cells at all time points studied (Fig. 19D).

In conclusion, one clear phenotype emerged. Ectopic expression of Brk inhibited cell survival in conditions of serum starvation and UV exposure combined with serum starvation. However, Brk overexpression didn’t change the growth characteristics of these cells. Activation of apoptosis pathways may be the most important function of Brk in its tumor suppressor properties.

4 Discussion

4.1. Regulation of intestinal homeostasis by Brk

The human and mouse genomes encode 32 non-receptor protein tyrosine kinases (PTKs) that mediate a vast number of very diverse, highly specific and finely regulated molecular events in the cells. Moreover, only a fraction of these PTKs are typically expressed in an individual cell or a specific tissue. This signaling diversity is achieved not only by the differential specificity of non-receptor PTKs, but primarily through the multitude of the interactions of non-receptor PTKs with multiple non-kinase proteins that can modify the effects of these PTKs and regulate their functions in the cell.

Brk is a novel, non-receptor tyrosine kinase involved in various signal transduction pathways. Although Brk shares approximately 44% sequence identity with Src, it belongs to a distinct family of Src-like intracellular tyrosine kinases (Serfas and Tyner, 2003). Like Src family kinases and the majority of signal transducers, Brk performs its biological function through interaction with other cellular components. This depends on multiple functional domains – the SH3 and SH2 protein binding domains as well as its tyrosine residues (Qiu and Miller, 2004). Furthermore similar to Src, the SH3 and SH2 domains of BRK engage in intramolecular interactions with the kinase domain to form an autoinhibited conformation (Qiu and Miller, 2002). Disruption of the autoinhibited conformation leads to BRK kinase activation and freeing of the SH3 and SH2 domains to recruit substrates or other interacting proteins. In contrast to Src tyrosine kinases, Brk lacks an amino-terminal myristoylation signal, and although an apparent nuclear localization signal is absent, this kinase is flexible in its intracellular localization. Brk is one of a few tyrosine kinases found in the nucleus (Derry, et al., 2000; Haegebarth, et al., 2004), and like c-Abl (Zhu and Wang, 2004), the intracellular localization of Brk may influence its protein-protein interactions and the signaling pathways that it regulates.

Brk expression is epithelial-specific and developmentally regulated (Llor, et al., 1999; Vasioukhin, et al., 1995). Brk is expressed throughout the skin and the gastrointestinal tract with highest levels in neonatal colon and adult ileum. Brk mRNA and protein expression are restricted to differentiating intestinal epithelial

cells, and it is excluded from proliferative cells of intestinal crypts, suggesting that Brk may play a role in the migration or terminal differentiation in these tissues (Vasioukhin, et al., 1995). Overexpression of Brk in mouse keratinocytes results in increased expression of the differentiation marker filaggrin during calcium-induced differentiation (Vasioukhin and Tyner, 1997), further supporting a role in differentiation for this kinase.

To begin to understand the biological role of Brk in the regulation of differentiation *in vivo*, mice carrying a disruption in the *brk* gene were generated. Since Brk is expressed at highest levels in the gastrointestinal tract it was chosen as the model system to characterize. The intestine is remarkable in that there is a high turnover of cells, arranged in order, undergoing proliferation in the crypt, and then differentiation to achieve the mature functions of absorptive enterocytes, mucus-producing goblet cells and enteroendocrine cells on the villus, or in case of the small intestine of Paneth cells at the base of the crypt (Sancho, et al., 2004).

Genetic ablation of *brk* in mice resulted in a range of low-penetrant phenotypes, including chronic inflammation (discussed in 4.2) and increased epithelial cell turnover in the gastrointestinal tract. Brk is not essential for embryonic development, as viable Brk null mice are obtained at the predicted Mendelian frequency in different genetic backgrounds. Homozygous deletions of the Brk family members Frk and Srms in mice have been generated as well (Chandrasekharan, et al., 2002, Kohmura, 1994 #2388), and similar to the Brk null mice, these homozygous mutants had no or only a mild phenotype. All mice were viable, fertile and not prone to developing spontaneous tumors. In contrast, homozygous deletion of Src42A in *Drosophila* caused lethality (Lu and Li, 1999). The lack of comparable effects for the individual mammalian Brk family tyrosine kinases suggests that they may share redundant functions. A high level of functional redundancy has been reported between Src family kinases. Evidence for compensatory changes in expression, activity, and subcellular localization of other Src family kinases has been reported in mice bearing single mutations, possibly accounting for the minimal phenotypes observed (Lowell, et al., 1996; Thomas, et al., 1995). Interestingly, the gastrointestinal tract expresses the highest endogenous levels of Brk and Frk (Sunitha and Avigan, 1996; Thuveson, et al., 1995; Vasioukhin, et al., 1995). It would be interesting to analyze effects of mutations in both Brk and Frk kinases on the

homeostasis of the gastrointestinal tract. Clearly, further investigations are needed to test this hypothesis.

However, Brk signaling seems to be required for maintaining the balance between proliferation and differentiation in the intestinal epithelium. Homeostasis of the intestinal epithelium strongly depends on the balance existing among cell proliferation, cell cycle arrest, cell differentiation, and cell migration (Simon and Gordon, 1995). Loss of Brk in mice resulted in the phenotypic appearance of longer villi and increased proliferation measured by BrdU incorporation and PCNA staining. Proliferation in the intestinal epithelium is usually restricted to cells in the lower base of the crypt compartment. However, in Brk knockout mice proliferating cells were present in the middle and upper regions of the crypt, where cells are normally fully differentiated and non-proliferating. This expanded proliferative zone and increased number of proliferating cells in Brk deficient mice suggests a deregulated balance in proliferation and differentiation in the absence of Brk signaling.

Deregulation of crypt homeostasis and the formation of aberrant crypt foci (ACF) is a feature of neoplastic transformation, and is evident in the earliest stage of colon cancer (Renehan, et al., 2002). In addition, it is appreciated that the process of apoptosis is vital for normal crypt homeostasis and its impairment may be an early event in the neoplastic process. In this work I present evidence that in normal tissue, such as the intestinal epithelium, Brk is required to restrict cell proliferation and promotes apoptosis in mice, features it shares with tumor suppressor proteins. Tumor suppressors normally act as inhibitors of cell proliferation or activators of apoptosis and use a variety of mechanisms in tissue growth suppression (Macleod, 2000; Sherr, 2004). Brk expression is restricted to non-proliferating cells in the intestinal epithelium, suggesting a function in differentiation. Loss of this tyrosine kinase in mice resulted in increased epithelial cell turnover, pro-survival signaling and chronic inflammation, features which have all been correlated with the promotion of cancer development (Philip, et al., 2004; Sancho, et al., 2004). Preliminary experiments utilizing the AOM mouse model of colon carcinogenesis confirmed this hypothesis. Whereas very few aberrant crypt foci (ACF) were visible in wild-type animals after treatment with the AOM carcinogen, knockout mice exhibited a high incidence of aberrant crypt foci formation (ACFs) within the distal colon (Heap and Bie, unpublished). Aberrant crypt foci are considered the smallest identifiable lesions

proposed to lead to colorectal carcinoma (Cheng and Lai, 2003), and their rapid appearance in Brk deficient mice strongly suggests a tumor suppressor function for Brk in the intestinal epithelium. However, due to the small number of animals used in these pilot experiments, further studies are currently underway.

Wnt signaling plays a major role in the regulation of intestinal cell proliferation and stem cell maintenance (Polakis, 2000; Sancho, et al., 2004), and its activation has been shown to result in hereditary and sporadic colon cancers (Bienz and Clevers, 2000). All intestinal malignancies share the characteristic of genomic instability and aberrant cell signaling pathways. One protein playing a crucial role in these diseases is the APC tumor suppressor protein. The APC protein is expressed throughout the intestine, where it functions as part of the Wnt pathway downregulating cytoplasmic levels of β-catenin (Hinoi, et al., 2000). Mutations in the *Apc* tumor suppressor gene initiate a majority of human colon cancers, and mice heterozygous for *Apc* mutations develop intestinal polyps. In APC-mutant mice, the wild-type allele is always lost in tumors. Inactivating mutations in APC have been placed at the beginning of the adenoma-carcinoma sequence in the development of colorectal cancer.

In search of signaling pathways being involved in the increased epithelial cell turnover in Brk deficient mice, the expression of β-catenin was analyzed. β-catenin in complex with members of the Lef/Tcf transcription factor family mediates a proliferation/ differentiation switch along the crypt-villus axis (Pinto, et al., 2003, van de Wetering, 2002 #3550) through activation/ repression of various target genes (Sancho, et al., 2004). These target genes play critical roles in the physiology of the intestine, as shown for the EphB2 and EphB3 in cell position in the intestine (Batlle, et al., 2002).

Loss of Brk resulted in nuclear accumulation of β-catenin not only in cells occupying basal positions of the crypt, as was observed in wild-type controls, but also in cells in higher positions of the crypt that usually lack nuclear β-catenin (Batlle, et al., 2002; van de Wetering, et al., 2002). Furthermore, this increased nuclear accumulation of β-catenin in the intestinal epithelium of Brk deficient mice resulted in increased expression of the β-catenin target gene *c-myc* in the intestinal crypts. c-Myc has been identified as one of the main mediators of the switch between

proliferation and differentiation along the crypt-villus axis (He, et al., 1998). Thus, the upregulated expression of *c-myc* in the intestine of knockout mice is most likely the molecular mechanism causing the increased proliferation. Furthermore, the c-Myc protein is also playing a central role in the proliferative capacity of many cancers, including colorectal carcinoma (Grandori, et al., 2000). Thus, loss of Brk expression and subsequent signaling promotes activation of the Wnt pathway, implying that the activation of Wnt signaling is responsible for the observed intestinal phenotype in knockout mice.

But by which mechanism does the Brk tyrosine kinase negatively regulate the expression of β-catenin? Interestingly, the loss of Brk resulted in activation of Akt in the intestine. Brk deficient mice exhibited significantly upregulated levels of Akt kinase activity when compared to wild-type controls. Supporting these data, analysis by Carol A. Lange and colleagues showed that Brk associates with Akt resulting in inhibition of Akt activity (Zhang, et al., 2004). Association of Akt with Brk results in tyrosine phosphorylation of Akt, which in turn has been shown to result in reduction of Ser473 phosphorylation and inhibition of Akt kinase activity (Conus, et al., 2002). Akt, in turn, has been shown to facilitate the stabilization and nuclear accumulation of β-catenin either indirectly through inhibition of GSK-3β or through direct phosphorylation of β-catenin (Fukumoto, et al., 2001; Persad, et al., 2001; Tian, et al., 2004). These data would leave us with the following model (Fig. 20): in normal intestinal epithelial cells Brk might be positioned to inhibit Akt kinase activity, resulting in the stabilization of GSK-3β, which in turn phosphorylates β-catenin contributing to the degradation of β-catenin. Loss of Brk on the other hand would result in increased Akt kinase activity and increased nuclear accumulation of β-catenin, thus contributing to increased epithelial cell turnover. However, it remains to be addressed as to how exactly Brk interacts with Akt. Furthermore, it has to be investigated whether Brk actually colocalizes with Akt in epithelial cells of the intestine.

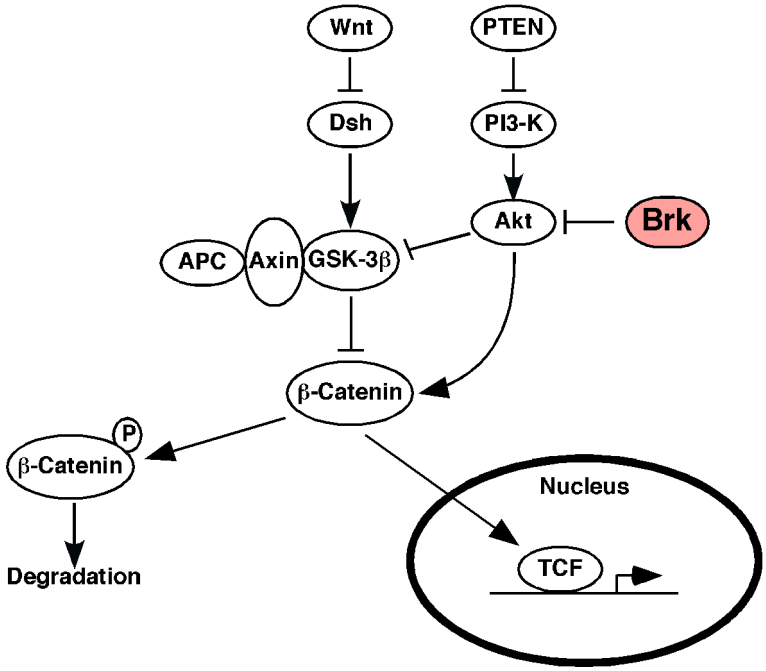

Fig. 20: Illustration of a putative regulatory role of Brk tyrosine kinase signaling. Brk represses Akt kinase activity, which is required to fully activate β-catenin. Thus, Brk inhibits nuclear-accumulation of β-catenin and transcription of β-catenin target genes in differentiated cells. Wnt signaling is also required but not sufficient to fully activate β-catenin.

4.2. Brk signaling and inflammation

The intestinal lining contains more lymphoid cells and produces more antibodies than any other organ in the body, and it has been proposed that approximately 25% of the intestinal mucosa consists of lymphoid tissue (Neutra, 1998). Intestinal lymphoid tissue is present both diffusively as intraepithelial lymphocytes, scattered lymphocytes and plasma cells in the lamina propria, and in organized mucosa-associated lymphoid tissue consisting of single or multiple lymphoid follicles known as Peyer's patches. Intense immunological interactions occur between these immune cells and intestinal epithelial cells, which are known to interact with mesenchymal cells, such as myofibroblasts, and immunologically active macrophages, dendritic cells and lymphocytes, both intraepithelial and in the lamina propria (Berin, et al., 1999).

The regulation of proliferation of the stem cells in intestinal crypts is critical for maintenance of the structure of the mucosa and the capacity for effective absorption and defensive barrier functions. Although the intestinal epithelium is only one cell thick, it is sealed by tight and adherens junctions, which exclude peptides and

antigenic macromolecules and mediate intercellular adhesion and “contact inhibition”. Brk wild-type and knockout mice exhibited no differences in expression of the tight junction marker ZO-1 and the adherens type junction marker β-catenin (membrane-bound) in the intestinal villus epithelium. However, loss of Brk expression resulted in the increased presence of Peyer’s patches in the gastrointestinal tract of outbred knockout mice compared with wild-type controls. Peyer’s patches are the sites for transport, processing and presenting of foreign antigens, which is a main part of the effective immune surveillance of the mucosal surface. Their high number suggests increased immune activity in the intestine of Brk knockout mice.

Furthermore, intestinal epithelial cells have been shown to act not merely as a passive barrier but also as sensitive indicators of infection that initiate defense responses. Noninvasive as well as invasive organisms have been demonstrated to elicit production of chemoattractants, suggesting that receptor-mediated signaling pathways may be involved. Epithelial cells can produce cytokines and chemokines that attract and activate immune cells with potentially important effects on the immediate and long-term host defense functions. For example, Paneth cells secrete potent antimicrobial molecules at the crypt base (Quellette, et al., 1989).

In addition to the observed increase in lymphoid tissue, loss of Brk expression resulted in the upregulated expression of the cytokines IL-6 and IL-18 in outbred mice (2.8). However, no differences in baseline cytokine expression were observed in inbred Brk deficient mice (2.8 G12). It has been reported that host genetic determinants play a major role in the sensitivity to inflammation and the following tumor risk (Rogers and Fox, 2004). In general, genetically engineered mice on a 129/Sv strain background appear to be especially susceptible to IBD-like disease. On the other hand, mice on a C57BL background are sometimes resistant to inflammation compared with equivalent mutant mice on other genetic backgrounds.

Cytokines are soluble mediators of cell-to-cell communication and their upregulation has been correlated to inflammation and cancer. Both, IL-6 and IL-18 have been shown to be involved in the immune response and are considered pro-inflammatory cytokines. IL-6 plays a major role in the acute phase response, B cell maturation and macrophage differentiation (Diehl and Rincon, 2002). It furthermore counteracts apoptosis and can promote cancer development. Increased IL-6 production has been shown to correlate with colon tumor formation and growth

(Becker, et al., 2004). IL-18 is a novel cytokine produced by various cells including intestinal epithelial cells. However, within the gut mucosa IL-18 is primarily produced by intestinal epithelial cells (Pizarro, et al., 1999). It has been shown to induce interferon-γ (IFN-γ), and to play a crucial role in proliferation and maintenance of the intestinal epithelium (Okazawa, et al., 2004). Increased levels of IL-18 have been found in intestinal mucosal biopsies from inflammatory bowel disease (IBD) patients (Monteleone, et al., 1999; Pizarro, et al., 1999).

Inflammatory bowel disease (IBD) denotes chronic inflammatory disorders of the gastrointestinal tract of unknown etiology that comprises 2 major groups: ulcerative colitis (UC) and Crohn's disease (CD). Deregulation of the intestinal immune system, both at the humoral and cellular level, constitutes an important element in the multifactorial pathogenesis of IBD (Rogler and Andus, 1998). The expression of pro-inflammatory cytokines, most notably IL-1, IL-6, IL-18, TNF-alpha, and immunoregulatory cytokines, such as IL-2 and IFN-γ, in intestinal mucosa from IBD patients is markedly enhanced; however, it is not always accompanied by increases in cytokines' serum levels. Patients with inflammatory bowel disease (IBD) have an increased risk of developing colorectal cancer (Langholz, et al., 1992). The release of pro-inflammatory cytokines by enterocytes has been shown to be a major part in the immune response, ensuring that cells are not only called into epithelial/mucosal sites from the circulation but are activated and sustained once they reach this site. For example, cytokines are important activators of local macrophages, which also participate in host protection against infection. Furthermore, these cytokines can themselves induce epithelial cells to produce chemokines so that the initial stimulus by a microorganism can have an important multiplier effect. Thus, the observed upregulation of cytokine production in Brk deficient mice indicates an increased immune activity and suggests the occurrence of inflammation in their intestinal epithelium. Up-regulation of both IL-6 and IL-18 expression in Brk deficient mice supports the hypothesis of increased inflammation in knockout mice as indicated by the large amount of Peyer’s patches.

Growth factors and cytokines, produced either by epithelial or immune cells, can positively or negatively regulate the replication of the epithelium. These interactions frequently involve members of the cytokine superfamily, and the signaling from these receptors is in turn linked to tyrosine kinases expressed in the

epithelium. Altered local cytokine production has been shown to be critical for inducing increased rates of epithelial cell turnover in inflammation (Podolsky, 2002). One could propose that the induced cytokine production through loss of Brk tyrosine kinase signaling in knockout mice further contributes to the observed phenotype of increased epithelial cell turnover. It furthermore has long been appreciated that small intestinal injury, for example in celiac disease, results in hypertrophy of the crypt compartment, but the mechanisms responsible for regulation of proliferation in the crypts have not been defined. However, it has also been proposed that inflammatory cytokines have anti-proliferative effects on epithelial cells (Booth and Potten, 2001; Neta and Okunieff, 1996).

The observation of increased epithelial cell turnover, high amount of lymphoid tissue and upregulated cytokine production in the intestine of mice deficient for Brk strongly suggests a role for this tyrosine kinase in maintaining intestinal homeostasis including a functional host defense barrier. Inflammation is the physiological response to injury caused by wounding, chemical irritation/damage, or infection. Whereas acute inflammation has been shown to lead to tumor regression and counteract cancer development, chronic inflammation leads to cellular proliferation thus strongly promoting cancer development (Philip, et al., 2004). Chronic inflammation has been shown to promote carcinogenesis indirectly in a non-cell-autonomous fashion. Upregulation of inflammatory mediators creates a tumor-promoting environment in which transformed epithelial cells thrive more optimally (Oshima, et al., 1996). Recently a more direct model has been proposed in which inflammatory signaling in the epithelial cells results in their inappropriate survival and transformation (Rakoff-Nahoum, et al., 2004, Greten, 2004 #3568). Thus, one could conclude that the apparent chronic inflammation in knockout mice predisposes these mice to cancer.

In addition to the reported baseline defects of immune response in Brk deficient intestinal epithelium, ablation of Brk signaling in the intestinal epithelium resulted in increased susceptibility to DSS, while the production of many inflammatory mediators was actually enhanced. Dextran sulfate sodium (DSS) has been shown to induce colitis by chemical injury in the intestinal epithelium accompanied by mild inflammation (Cooper, et al., 1993; Okayasu, et al., 1990). After subjecting wild-type and Brk knockout mice to this irritant, the mutant

epithelium appeared more susceptible to DSS-induced histological damage than control epithelium. DSS treatment caused severe and acute inflammation accompanied with severe loss of epithelium in Brk deficient mice. Brk mutant mice showed severe colonic erosions, loss of crypts and an extent amount of inflammatory infiltrate in their submucosa and lamina propria following DSS treatment. In addition to the histological differences, Brk deficient mice exhibited an elevated expression of pro-inflammatory cytokines, such as IL-1 and IL-6, suggesting the occurrence of acute inflammation in these tissues. Acute inflammation is a self-limiting process that starts a cascade of cytokines and chemokines that attract immune and non-immune cells, mainly neutrophils, to infiltrate disrupted and damaged tissue. However, inflammation, even though to a much lesser degree, is also observed in wild-type animals. Furthermore, loss of IL-18 expression was observed in mutant mice. IL-18 is mainly produced by epithelial cells in the intestinal mucosa and its loss further confirmed the overall loss of intestinal epithelium in Brk deficient mice after treatment. Thus, Brk signaling seems to be required for epithelial cell integrity and loss of Brk signaling results in increased epithelial susceptibility to injury.

Interestingly, Brk protein expression was upregulated in the intestinal epithelium after DSS treatment. Nuclear Brk protein was present not only in differentiating cells but also in the proliferating cells of the crypt epithelium. This induction of Brk protein strongly suggests a role for Brk tyrosine kinase signaling in the protection against DSS-induced injury. Brk may protect the intestinal epithelium against injury by ensuring epithelial homeostasis. Consistent with this notion is the increased proliferation of intestinal epithelial cells in Brk deficient mice. This by itself would make them more susceptible to damage (Booth and Potten, 2001; Neta and Okunieff, 1996). The exact molecular mechanism of this effect remains to be detailed. Furthermore, upregulation of Brk protein expression might influence the steady-state production of protective factors, such as COX-2 or TGF-β1, which have been shown to be crucial in protecting the gut from injury (Dignass, 2001; Podolsky, 1999). However, it needs to be delineated, whether upregulation of nuclear Brk upon epithelial damage may actually induce the expression and production of cytoprotective factors. It has long been appreciated that mesenchymal-epithelial crosstalk and following cell signaling is crucial to the orchestration of responses to tissue injury (Clark, 2003).

Of special interest is the strong nuclear expression of Brk throughout the intestinal epithelium in response to DSS. Due to the lack of an amino-terminal myristoylation signal, the intracellular localization of Brk is flexible and it is one of a few tyrosine kinases found in the nucleus (Derry, et al., 2000; Haegebarth, et al., 2004). Nuclear localization of tyrosine kinases has been shown to be important for connecting extracellular signaling with the direct regulation of gene expression. Signal tranduction to the nucleus and the subsequent regulation of gene expression is of main importance for cells to react to external stimuli (Brivanlou and Darnell, 2002). Furthermore, tyrosine phosphorylation of nuclear proteins regulates many cellular processes, including growth, differentiation, and apoptosis (Cans, et al., 2000; Wang, 2000). Thus, Brk's nuclear localization provides a means to direct communication between the cytoplasm and the nucleus, and even provides a platform for the integration of external signals. It is of importance to identify how this tyrosine kinase shuttles between cellular compartments and if its nuclear localization is necessary in response to growth factors. Furthermore, nuclear localization of PTKs, which possess various protein-protein binding domains, can also support the transport of other molecules to the nucleus. A similar function has been proposed for ErbB-1 in transporting STAT-1, a tyrosine-phosphorylated transcription factor, to the nucleus (Bild, et al., 2002). However, evidence supporting this notion for the Brk non-receptor tyrosine kinase needs to be found.

Non-receptor tyrosine kinases, such as Frk, have been found in the nucleus, but relevant substrates are still unknown or poorly understood (Cance, et al., 1994, Derry, 2000 #2416; Haegebarth, et al., 2004). One of the first Brk substrates identified was the RNA-binding protein Sam68 (Derry, et al., 2000). Brk has been shown to phosphorylate Sam68 in the nucleus resulting in the inhibition of its RNA binding and transport functions. Several studies support roles for Sam68 in the regulation of RNA metabolism and utilization (McLaren, et al., 2004; Reddy, et al., 1999; Soros, et al., 2001). Interestingly, Sam68 has also been found to colocalize and associate with RNA splicing factors, thus regulating alternative splicing (Denegri, et al., 2001; Hartmann, et al., 1999; Matter, et al., 2002). More importantly, this regulation of alternative splicing, and therefore gene expression, is dependent on extracellular signaling through Erk kinases (Matter, et al., 2002). It can be hypothesized that nuclear localization of Brk, and the subsequent phosphorylation and

inhibition of its nuclear substrate Sam68, might influence splicing associated functions of Sam68 and hence regulate gene expression.

The increased susceptibility of Brk deficient mice to inflammation and injury was further supported by the results of the DSS/AOM colitis-associated tumor model reported in this study. Wild-type and Brk deficient mice were subjected to a single injection of AOM followed by a 1-week treatment with 2% DSS in drinking water. This protocol has been shown to effectively and specifically induce colonic adenocarcinoma and colitis in a relatively short time (Tanaka, et al., 2003). It is well known that increased cell turnover and chronic inflammation promotes and greatly predisposes to cancer development (Itzkowitz and Yio, 2004; Sancho, et al., 2004). Since loss of Brk resulted in increased epithelial cell proliferation and chronic inflammation in the intestine, it was hypothesized that Brk deficient mice would be more prone to tumor development in a mouse colon tumor model, further supporting putative tumor suppressor functions of Brk in the gastrointestinal tract. This was confirmed in a pilot study utilizing the traditional AOM mouse model of colon carcinogenesis, where mice receive weekly AOM injections for the duration of 6 weeks and are sacrificed 4 or 24 weeks after the last injection (Boivin, et al., 2003). Ten weeks after subjecting wild-type and knockout mice to the AOM pro-carcinogen, knockout mice exhibited a considerably higher incidence of aberrant crypt foci (ACF) formation within the distal colon compared to wild-type controls (Heap and Bie, unpublished). At 30 weeks after AOM treatment, knockout mice developed colonic tumors, which were absent in wild-type controls.

In stark contrast to this study were the results obtained in the accelerated DSS/AOM tumorigenesis model. In this carcinogenesis model Brk deficient mice showed a reduced colitis-associated tumor incidence when compared to wild-type animals. Furthermore, wild-type mice showed increased mortality upon treatment. Combination of the initial injection of the carcinogen AOM with 2% DSS treatment to induce acute inflammation and thus promoting tumor development actually had the opposing effect in knockout animals. The reason for these seemingly paradoxical results lies in the response to the DSS treatment itself. Previously an immediate acute inflammatory response with severe loss of crypt epithelium to DSS treatment has been observed in Brk deficient mice. Thus, the cells carrying oncogenic mutations caused by AOM might actually be ablated during the process of acute inflammation

resulting in the lower tumor incidence observed in Brk deficient mice. In wild-type mice on the other hand, after the initial low dose of AOM, tumor formation is promoted by the induction of inflammation through treatment with 2% DSS. The "AOM-initiated cells" carrying oncogenic mutations are supported by the inflammation driving these mutant cells to proliferate and giving preneoplastic and neoplastic cells a growth advantage. This hypothesis may explain the high tumor incidence in wild-type mice whereas Brk deficient mice are seemingly more resistant to tumor formation. Due to the increased susceptibility of Brk deficient mice to inflammation and injury, the accelerated DSS/AOM tumorigenesis model appears to be a “bad” choice to confirm putative tumor suppressor functions for Brk in the intestine. To test this hypothesis, further studies with lower doses of DSS (0.5%) as well as the long-term “AOM only” tumor model are underway.

Our findings reveal a new role for Brk tyrosine kinase signaling in the maintenance of epithelial homeostasis and protection from intestinal injury. Brk kinase signaling might play a crucial role in signaling pathways involved in the response to injury. Exactly which pathways Brk interacts with remains to be determined.

4.3. Regulation of apoptosis by Brk signaling

The intestinal epithelium is a rapidly renewing tissue in which cells undergo topographically organized proliferation and differentiation. Production of epithelial cells occurs in the crypts, which then differentiate and migrate toward the apex of the villus. Eventually, cells are shed at the tip of the villus or the surface epithelium. This cell loss is precisely balanced in steady state by cell division occurring in the crypt compartment. Intensive studies of cell kinetics of this system revealed that approximately 1000 cells are being shed per small intestinal villus each day. It has been shown that apoptosis or programmed cell death is required for maintaining intestinal tissue homeostasis, the defense against certain pathogens and elimination of unwanted cells (Vachon, et al., 2001; Vachon, et al., 2000). Furthermore, mutations affecting critical genes that regulate cell proliferation and survival cause fatal cancers.

As previously discussed, loss of Brk results in increased epithelial cell turnover and the physiological appearance of longer villi in the gastrointestinal tract

of mice. Apoptosis is a crucial mechanism required to counterbalance the crypt cell division (Potten, 1992), and the appearance of longer villi in Brk deficient mice suggested an impaired balance between cell production and cell loss. In keeping with this hypothesis, no statistically significant differences in spontaneous apoptosis within the crypt epithelia of untreated wild-type and Brk knockout mice were observed. However, Brk deficient mice showed severe inability to respond to genotoxic stress, such as radiation, suggesting a role for Brk in DNA-damage induced apoptosis. Cellular damage can initiate apoptotic cell death, both *in vitro* and *in vivo*. Whether a cell will survive or undergo apoptosis after DNA damage greatly depends on patterns of gene expression determining a survival threshold and modulating the engagement of apoptosis (Dive and Hickman, 1991, Salvesen, 1997 #3488). The crypts of the small intestine provide a readily accessible and quantifiable model to study the apoptotic response to genotoxic damage *in vivo* (Merritt, et al., 1997). Following DNA-damage, the stem cells in the small intestinal crypt are committed to an altruistic cell suicide, which is required to avoid the accumulation of oncogenic mutations, resulting in the protection against cancer (Potten, 1992).

Whole-body γ-irradiation of mice with doses of 8 Gγ cause p53-dependent apoptosis in the small intestinal crypts with 6 apoptosis-susceptible cells in each crypt (Potten, 2004). Wild-type and Brk deficient mice were subjected to whole-body γ-irradiation and the amount of apoptotic cells was microscopically identified by caspase 3 activation. Interestingly, Brk deficient mice were more resistant to apoptosis induced by γ-irradiation than their wild-type counterparts. They exhibited a twofold reduction in their response to radiation, with only 2-3 apoptotic cells in each crypt. In addition, after ionizing radiation, induction of Brk protein was observed in wild-type mice. After DNA damage, Brk expression was not only detected in non-proliferating cells of the villus but also in the crypt compartment of the intestinal epithelium, which usually excludes expression of this tyrosine kinase. These data support the notion that Brk is required for the altruistic cell suicide of the crypt stem cell following DNA damage. Thus, Brk is not only protecting the small intestine against injury, as has been shown in the DSS model, but also against oncogenic mutations caused by irradiation. These facts support a tumor suppressor function for Brk in the intestine, since loss of this kinase results in impaired apoptosis.

Brk signaling might contribute to the induction of apoptosis by regulating the PI3-kinase/Akt survival pathway. Brk has been shown to phosphorylate and inhibit Akt kinase activity and downstream signaling in unstimulated cells (Zhang, et al., 2004). Furthermore, untreated Brk deficient mice exhibited increased Akt kinase activity. Similar to untreated conditions, Brk mutant mice showed constitutively higher levels of phospho-Ser473 Akt and Akt kinase activity after γ-irradiation when compared to wild-type controls. The serine/threonine kinase Akt has been reported to mediate cell survival by various growth factors and cytokines in a variety of cell types and to block and protect against apoptosis induced by multiple apoptotic stimuli (Aikawa, et al., 2000; Datta, et al., 1999; Fujio, et al., 2000; Kandel and Hay, 1999; Matsui, et al., 1999). Brk seems to be acting as a repressor of Akt activity, thus regulating growth and survival in the intestinal epithelium. Negative regulation of Akt, as shown in the case of the PTEN phosphatase tumor suppressor, results in the promotion of apoptosis, whereas loss of this repression has been implicated in a wide range of human cancers (Haas-Kogan, et al., 1998; Li, et al., 1998; Stambolic, et al., 1998; Wu, et al., 1998). The observed induction of Brk protein expression in the crypt stem cells of wild-type animals after irradiation, together with increased Akt kinase activity in Brk deficient mice, suggest that repression of Akt pro-survival signaling by Brk in the crypt might be one mechanism contributing to DNA-damage induced apoptosis in the intestine.

Furthermore, loss of Brk resulted in increased phosphorylation and therefore activation of extracellular signal-regulated kinases (Erk1/2) following irradiation. Differential involvement of the Erk signaling pathways in the regulation of epithelial cell survival through regulation of balanced transcription of anti-apoptotic and pro-apoptotic genes has been reported (Gauthier, et al., 2001; Gauthier, et al., 2001). Activation of Erk1/2 has been shown to promote cell survival, whereas activation of the stress-activated c-Jun N-terminal kinases ($p46/p54^{JNK}$) and $p38^{MAPK}$ induces apoptosis (Wang, et al., 1998; Wang, et al., 1998; Xia, et al., 1995). The activation of Erk kinases in Brk deficient mice, together with activated Akt kinase activity, indicates that these pro-survival signaling pathways might contribute to the resistance of Brk deficient mice against apoptosis. Brk signaling appears to be required for the induction of altruistic cell death upon cellular or DNA damage. Therefore, signaling mediated by this tyrosine kinase is protecting the integrity of the small intestine

against genetic alterations that would otherwise cause cell transformation and the development of cancer. This is confirmed by the previously discussed AOM tumorigenesis pilot study, in which Brk deficient mice showed increased formation of ACFs (Heap and Bie, unpublished). The resistance of Brk mutant mice to apoptosis, and the resulting continued survival of cells with DNA damage and/or oncogenic mutations, presents the potential for carcinogenic transformation. Thus, Brk deficient mice would be more prone to tumor development, supporting putative tumor suppressor functions of Brk in the gastrointestinal tract.

It should also be noted, that Brk can shuttle between the nuclear and cytoplasmic cell compartments, and therefore could conceivably transmit the DNA damage signal from the nucleus to the cytoplasm. This has been shown to be the fact for the pro-apoptotic function of the c-Abl tyrosine kinase (Taagepera, et al., 1998). In other words, upon DNA-damage nuclear Brk tyrosine kinase may be activated and exit the nucleus, resulting in further inhibition of Akt kinase activity and thus pro-survival signaling.

In addition to these *in vivo* observations, Brk is also involved in regulating apoptosis in an *in vitro* model system. Overexpression of Brk in Rat1A fibroblasts sensitizes these cells to apoptosis induced by serum starvation or a combination of UV irradiation/ serum starvation *in vitro*. Rat1a cells are immortal nontransformed rat embryo fibroblasts that are susceptible to oncogenic transformation and exhibit serum- and Akt-dependent susceptibility to a variety of apoptotic stimuli (Topp, 1981). Increased susceptibility to apoptosis of fibroblasts expressing active Brk further supports a significant role for Brk tyrosine kinase in the regulation of the apoptotic response to DNA-damage. In addition, apoptosis in these fibroblasts is dependant on Akt signaling, and therefore it would be interesting to explore if the introduction of dominantly active Akt into Brk stable Rat1A fibroblasts is able to rescue cells from Brk-mediated apoptosis.

4.4. Brk signaling in tumor suppression and cancer development

Tumor suppressors normally act as inhibitors of cell proliferation or activators of apoptosis and use a variety of mechanisms in tissue growth suppression (Macleod, 2000; Sherr, 2004). Brk is positioned to inhibit cell proliferation by its restriction to

differentiated cells in the small intestinal villus or colon. Loss of Brk resulted in increased epithelial cell turnover most likely due to deregulated Wnt signaling. Increased accumulation of nuclear β-catenin and upregulation of the β-catenin target gene *c-myc* were observed in Brk deficient mice. In addition, these mice exhibited increased pro-survival signaling in terms of increased Akt kinase activity. Brk has been shown to inhibit Akt kinase activity *in vitro* (Zhang, et al., 2004), and thus it is conceivable that loss of Brk results in increased Akt activity in the intestine. This in turn, could contribute to the increased nuclear accumulation of β-catenin in Brk deficient mice. It could be proposed that Akt activation and β-catenin signaling can be effectively controlled by the availability and activity of Brk protein through differential distribution of Brk in various tissues, cells, or subcellular localization. However, co-localization of Brk with Akt in intestinal epithelial cells still needs to be confirmed.

Furthermore, Brk deficient mice exhibited chronic inflammation, which, as shown in inflammatory bowel disease, significantly increases the risk of developing intestinal cancer. When subjected to DSS, a colon injury model, Brk deficient mice undergo severe epithelial injury and cell loss. This could in part be contributed to the deregulated intestinal tissue homeostasis observed in Brk deficient mice. In this injury model, wild-type animals showed significant upregulation of Brk protein levels and strong nuclear expression of this tyrosine kinase throughout the intestinal epithelium in response to DSS, suggesting a role for Brk in protecting the epithelium from injury. In addition, mice carrying a loss of function mutation in the *brk* gene are more resistant to DNA damage induced apoptosis in the small intestinal crypt. No differences in base-line apoptosis were observed in untreated animals. However, when subjected to γ-irradiation, Brk deficient animals are significantly more resistant. Thus, these mice are impaired in the altruistic cell suicide, a crucial mechanism protecting the stem cells in the small intestinal crypt against the accumulation of oncogenic mutations and therefore cancer (Potten, 1992). Wild-type mice on the other hand, displayed apoptosis in response to irradiation at levels consistent with the literature (Potten, 2004). In addition, they exhibited a rapid radiation-induced upregulation of Brk protein levels and expression of Brk in crypt cells, which usually exclude its presence. These data suggest that Brk functions as a determinant of cellular sensitivity to genotoxic stress in the intestinal epithelium.

Overall one could propose, that in normal tissues such as the intestinal epithelium, Brk has tumor suppressor functions such as the regulation of differentiation and cell cycle exit and the protection of the epithelium against injury and oncogenic mutations (Fig. 21).

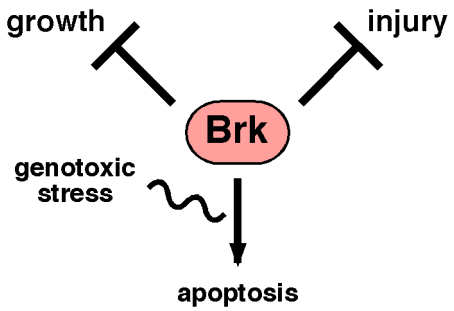

Fig. 21: Brk tumor suppressor qualities. Brk is required for intestinal homeostasis. It is positioned to inhibit cell proliferation in the intestinal epithelium. Loss of Brk results in increased epithelial cell turnover and chronic inflammation in the intestine. In addition, Brk protects the intestine against injury and oncogenic mutations. The Brk tyrosine kinase is required for the induction of intestinal apoptosis following genotoxic stress.

However, in contrast to the gastrointestinal tract, Brk is expressed in a significant proportion of primary breast tumors and breast tumor cell lines, although it is not expressed in normal mammary gland epithelial cells (Barker, et al., 1997). Furthermore, Brk protein was detected in metastatic melanoma cell lines (Easty, et al., 1997), as well as head and neck squamous cell carcinomas (Lin, et al., 2004), and it is capable of promoting cell motility, migration and invasion through phosphorylation of paxillin (Chen, et al., 2004). These seemingly paradoxical roles of Brk during differentiation and tumorigenesis are still poorly understood. However, protein mislocalization has been shown to play an important role in the development of cancer and has been observed for a number of signaling proteins (Kau, et al., 2004). Differential intracellular localization of Brk has been observed in several epithelial cancers of varying malignancy grades (Derry, et al., 2003; Derry, et al., 2000; Petro, et al., 2004; Serfas and Tyner, 2003). Thus, the ability of Brk to associate with distinct sets of substrates in different cellular compartments in normal tissues and cancer cells may lead to the activation of divergent signaling pathways. Its role in biology is likely to be determined by its relative kinase activity in coordination with its interaction with other proteins as part of regulated signaling complexes. While

functions of most Brk family kinases are poorly understood, a common feature of Brk family proteins is to limit receptor kinase signaling in untransformed cells (possibly by functioning as co-inhibitors of Akt or inhibitors of Ras pathway signaling) (Lu and Li, 1999; Zhang, et al., 1999), or during differentiation of skin or gut epithelial cells (Anneren and Welsh, 2000; Oberg-Welsh, et al., 1998; Vasioukhin, et al., 1995; Vasioukhin and Tyner, 1997). In cancer cells on the other hand, association of these kinases with de-regulated signaling complexes might contribute to cancer development.

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Eidestattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig und nur unter Verwendung der angegebenen Hilfsmittel erarbeitet und verfasst habe. Diese Arbeit wurde keiner anderen Prüfungsbehörde vorgelegt.

Chicago, 10. April 2005

Andrea Hägebarth